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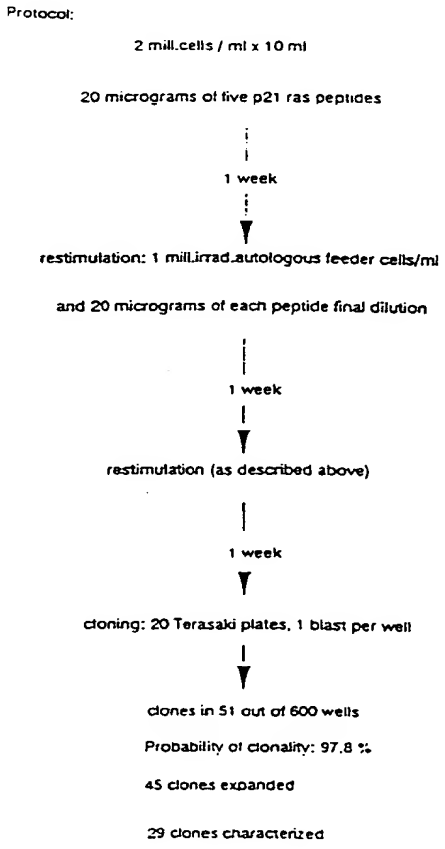
(54) Title: THERAPEUTICALLY USEFUL PEPTIDES AND PEPTIDES FRAGMENTS

(57) Abstract

Synthetic peptides and fragments of oncogene protein products which elicit T cellular immunity, for use in cancer vaccines and compositions for anti-cancer treatment.



THERAPEUTICALLY USEFUL PEPTIDES AND PEPTIDE FRAGMENTS.

Summary of the invention

This invention relates to synthetic peptides and fragments of oncogene protein products which elicit T cellular immunity, and to cancer vaccines and compositions for anti-cancer treatment comprising said peptides or peptide fragments.

Prior art

From EP272321 it is known to use an oncogene protein product or a fragment thereof for the production of an immunoglobulin specific for the oncogene protein, and thereafter forming a conjugate of said immunoglobulin and an anti-cancer agent to be used in the treatment of cancer.

From EP177814 and EP175360 it is known to produce antibodies against oncogene protein products, such as the p21 protein of ras, only differing in one amino acid in position 12 from the normal protein product. These antibodies may be used for diagnostic or therapeutic purposes. In order to achieve immunogenic peptide fragments of the p21 ras protein, a cysteine residue is inserted between the positions 16 and 17, to which a protein carrier may be attached.

Further, from EP 253325, there are known oncogene-related peptides, which comprise a portion of the amino acid sequence coded for by an oncogene, and antibodies directed to said peptides.

It is further known that one approach to an immunological cancer therapy has been through administration of interleukin-2 combined with specific lymphocytes, so called lymphokine-activated killer cells (LAK cells), or tumour

are known to recognize different, non overlapping epitopes of the same protein. The explanation for this is thought to reside in differences between the species in their antigen processing machinery and peptide binding capabilities of their MHC molecules.

From Stefan Jung and Hermann J. Schleusener, J. Exp. Med., Vol. 173, Jan. 1991 it is reported that a synthetic peptide fragment of the amino acids 5-16 of the p21 ras protein having a valine instead of a glycine amino acid in position 12 is recognized by human $CD4^+$ T cells from two healthy persons and that these T cells may be generated as antigen specific T cell lines, which do not cross react with the corresponding peptides derived from the normal p21 ras proteins. In this work it is shown that the human immune system recognizes this single synthetic peptide fragment.

The relevance of this finding is, however unclear since it is known that T cell reactivity against synthetic peptides may differ from T cell reactivity against the whole protein from which the peptides were derived. The explanation for this discrepancy being that equivalents of the synthetic peptide are not formed during proteolytic cleavage/processing of the protein in vivo. Thus, it is of vital importance that the peptide fragment used will elicit specific T cell responses or evoke memory T cell reponses to the actual oncogene protein fragment produced by processing and presented by the cancer cell and other antigen presenting cells. The definition of such peptides is a prerequisite for development of cancer vaccines and cancer therapy based on T cell immunity.

Technical Background

The genetic background for the onset of cancer are proto-oncogenes and oncogenes. Proto-oncogenes are normal genes of

Antibodies typically recognize free antigen in native conformation and can potentially recognize almost any site exposed on the antigen surface. In contrast to the antibodies produced by the B cells, T cells recognize antigens only in the context of MHC molecules, designated HLA (human leucocyte antigen) in humans, and only after appropriate antigen processing, usually consisting of proteolytic fragmentation of the protein, resulting in peptides that fit into the groove of the MHC molecules. This enables T cells to recognize also peptides derived from intracellular proteins. T cells can thus recognize aberrant peptides derived from anywhere in the tumor cell, in the context of MHC molecules on the surface of the tumor cell, and subsequently can be activated to eliminate the tumor cell harbouring the aberrant peptide.

The HLA molecules are encoded by the HLA region on the human chromosome No 6. The class I molecules are encoded by the HLA A, B and C subloci, and the class II molecules are encoded by the DR, DP and DQ subloci. All the gene products are highly polymorphic. Different individuals thus express distinct HLA molecules that differ from those of other individuals. This is the basis for the difficulties in finding HLA matched organ donors in transplantations. The significance of the genetic variation of the HLA molecules in immunobiology is reflected by their role as immune-response genes. Through their peptide binding capacity, the presence or absence of certain HLA molecules governs the capacity of an individual to respond to peptide epitopes. As a consequence, HLA molecules determine resistance or susceptibility to disease.

T cells may control the development and growth of cancer by a variety of mechanisms. Cytotoxic T cells, both HLA class I restricted CD8+ and HLA Class II restricted CD4+, may directly kill tumour cells carrying the appropriate tumour antigens. CD4+ helper T cells are needed for cytotoxic T cell

and

c) induce specific T cell responses to the actual oncogene protein fragment produced by the cell by processing and presented by the HLA molecule.

Since these mutations give rise to the transforming capacity of oncogenes, they are pivotal in the development of cancer. By raising specific T cell responses against these mutations control of the development and growth of the tumour cells carrying the mutation is possible. Thus for the first time it is possible to develop prophylaxis and therapy directed against the specific genetic alterations in neoplastic cells.

Detailed Description of the Invention

One purpose of the present invention is to develop a vaccine to prevent the establishment of cancers carrying the most commonly observed oncogene mutations, based partly or solely on synthetic peptides or peptide fragments of oncogene proteins which produce T cell immunity against the oncogene's gene product.

Another purpose of the present invention is to produce a cancer therapy for cancers having the said mutations in their proto-oncogenes, based on the T cell immunity which may be induced in patients by stimulating their T cell immunity either in vitro or in vivo with the peptides according to the present invention.

In order for a cancer vaccine and methods for specific cancer therapy based on specific T cell immunity to be effective, three conditions must be met:

1. The peptides used must correspond to, completely cover and/or be an active fragment of the processed oncogene protein fragment as presented by the cancer cell or other

Embodiments

The peptides according to this invention are synthetic peptides corresponding to and/or encompassing the processed peptide being presented by the cancer cell or other antigen presenting cells, including a mutation in one or more positions corresponding to the oncogene mutation, and giving rise to T cell immunity against the oncogene protein. The amino acid at the position of the point of mutation may be any amino acid except the amino acid found in the normal proto-oncogene encoded protein, but preferred are the amino acids found in oncogene proteins.

The peptides according to this invention further include fragments having one or several amino acid substitutions at the flanks of the point of mutation or translocation.

Transforming ras genes are the oncogenes most frequently identified in human cancer, with an overall incidence estimated to be around 10-20%. The transforming genes carry mutation in position 12, 13 and 61 in the ras gene product p21.

According to one aspect of this invention, the synthetic peptides are fragments including at least one of the positions 12, 13 and 61 of the oncogene protein ras p21, having the same amino acid sequence.

The amino acid in the position 12 may be any amino acid except Gly, which is found in the protein product encoded for by the proto-oncogene, when the reminder of the sequence corresponds to the normal proto-oncogene. One group of preferred peptides according to this invention are the peptides p113 - p119, the amino acid sequences of which will appear from Table 8, or fragments thereof, which will elicit a T cell response against the oncogene's protein product.

The amino acid in the position 13 may be any amino acid

are joined.

Other peptides are peptide fragments of a bcr-abl fusion protein, where exon c3 of bcr and exon 2 of abl are joined.

Preferred peptides of this group will comprise the following fragments or parts thereof:
Ile-Pro-Leu-Thr-Ile-Asn-Lys-Glu-Glu-Ala-Leu-Gln-Arg-Pro-Val-Ala-Ser-Asp-Phe-Glu

Ala-Thr-Gly-Phe-Lys-Gln-Ser-Ser-Lys-Ala-Leu-Gln-Arg-Pro-Val-Ala-Ser-Asp-Phe-Glu

Ala-Phe-Asp-Val-Lys-Ala-Leu-Gln-Arg-Pro-Val-Ala-Ser-Asp-Phe-Glu

Still other preferred peptides are fragments of ret fusion protein, comprising the following sequence or parts thereof:
Leu-Arg-Lys-Ala-Ser-Val-Thr-Ile-Glu-Asp-Pro-Lys-Trp-Glu-Phe

Still other preferred peptides are fragments of the EGF receptor fusion protein comprising the following sequence or parts thereof: Ser-Arg-Ala-Leu-Glu-Glu-Lys-Lys-Gly-Asn?-Tyr-Val-Val-Thr-Asp-His-Gly

Still other preferred peptides are fragments of the retinol receptor fusion protein comprising the following sequence or parts thereof: Leu-Ser-Ser-Cys-Ile-Thr-Gln-Gly-Lys-Ala-Ile-Glu-Thr-Gln-Ser-Ser-Ser-Ser-Glu-Glu

The present invention further includes larger fragments carrying a few amino acid substitutions at either the N-terminal or the C-terminal end, as it has been established that such peptides may give rise to T cell clones having the appropriate specificity.

immunity,

(3) the peptides may be targeted for a particular type of T cell response without the side effects of other unwanted responses.

The peptides or fragments according to the present invention can be included in pharmaceutical compositions or in vaccines together with usual additives, diluents, stabilizers or the like as known in the art.

According to this invention, a pharmaceutical composition or vaccine may include the peptides or fragments alone or in combination with at least one pharmaceutically acceptable carrier or diluent.

Further a vaccine composition can comprise a selection of peptides having the most common mutations as found in oncogene proteins.

Further a vaccine composition can comprise a peptide selected for one cancer, which vaccine would be administered to persons belonging to a high risk group for this particular cancer.

The peptides and peptide fragments according to this invention may be produced by conventional processes as known in the art, and this is elucidated in the description of the synthesis below.

As mentioned above a cancer vaccine according to the present invention may be administered to persons belonging to a high risk group for one definite cancer connected to one or several oncogenes. Examples of oncogenes found in human tumours will appear from Table 5.

The cancer vaccine according to this invention may further be

PBMC as feeder cells, as well as peptides and IL-2 as above, in a final volume of 120µl microlitre. Growing clones were further expanded in 24 well plates on day 3-5 after transfer from 96 well plates, in the same manner as described above, and restimulated weekly with fresh irradiated feeder cells, peptides and IL-2. Further expansion of the clones was done in medium containing only IL-2, and finally in medium without peptides or IL-2.

Figures 2a-2e show the specificity of the T cell clones I, B, E and F in T cell proliferation assays. Assays were set up in triplicate in 96 well plates, with the five peptides present in the original peptide mixture as stimulating antigens. The wells contained 50000 irradiated (8000 R) autologous EBV transformed B cells as antigen presenting cells (APC).

Cultures were incubated for 2 days at 37 ^{o}C in a 5 % CO2 incubator, and pulsed overnight with 1 µCi of ^{3}H-thymidine (Amersham, England) per well before harvesting onto glass fibre filters by an automated cell harvester (Scatron, Lierbyen, Norway). Thymidine incorporation into DNA was quantitated by liquid scintillation counting using an LKB 1205 Betaplate Liquid Scintillation counter. Data are given as medians of triplicate cultures. Controls included T cell clones cultured alone, or with APC in the absence of peptides. The panels are scaled according to the magnitude of the positive response.

Figure 3a-3d show the results of blocking experiments with clones I, B, E and F using the monoclonal antibodies L243, specific for HLA-DR and FN81.1.1, specific for HLA-DQ. Proliferation assays were as described in Figure 2, with the exception that the APC were preincubated for 30 minutes at 37 ^{o}C with the indicated concentration of monoclonal antibody before the addition of T cells and peptides to the incubation mixture. Data are expressed as described in Figure 2.

Figure 9 shows the results of stimulation of PBMC from a patient with follicular thyroid carcinoma with the whole panel of p21 ras derived peptides. PBMC, 100000 cells/well in 96 well plates were incubated with 100 µg/ml of each peptide in the absence of (first column) or presence of (second column) 1 U/ml of recombinant human IL-2. ^{3}H-thymidine (1 µCi) was added on day 6, and the cultures harvested and processed on day 7 as described in Figure 2. Results are given as medians of triplicates. Controls were PBMC cultured alone or in the presence of recombinant IL-2.

Figure 10 shows the response of PBMS from the same donor as in Figure 9 to peptide 23, in the presence or absence of IL-2. Conditions are as in Figure 9, except that 200000 cells per well was used, as well as two different doses of peptide.

Figure 11 shows the response of a secondary T cell culture derived from the patient described in Figure 9 and 10 to five peptides representing the amino acid sequence around position 61 of the normal and mutated p21 ras proteins.

Conditions were as described in Figure 2, except that 25000 of the responder cells abd APC were used, and the peptide concentration was 200 µg/ml.

Figure 12 shows the reactivity of the T cell clones 10, 14, 15, and 23 towards peptide 23. Conditions of the assay were as described in Figure 2, except that 50000 irradiated allogeneic HLA-DQ identical PBMC were used as APC, and peptide concentration was 50 µg/ml.

Figure 13 shows the results of blocking experiments with clone 14, using a monoclonal antibody FN81.1.1., specific for HLA-DQ. Conditions were as described in Figure 2 and 3.

Figure 14 shows the response of clone 15 to truncated forms of peptide 23. The peptides were truncated from the N-

Figure 19 shows the response of two T cell clones KB 15 and KB 23 derived from a healthy donor after repeated stimulation with a mixture of long peptides, (p-112, p-113, p-114, p-115 and p-116)) Culture conditions were essentially as described in Figure 1.

Figure 20 shows the results of growth inhibition studies of IFN-γ treated HT29 colon carcinoma cells (ATCC, Rockville MD) using clone 14 as effector cells. The number of target cells seeded per microwell was 20 000, and the cells were treated with recombinant human IFN (Amersham, UK), 500 U/ml for 3 days before the addition of irradiated (2000 rad) effector cells as indicated in the Figure. Peptide treated cells were cultured for the last 24 hours with peptide 106 at the final concentration of 10 μg/ml. After addition of effector cells, cultures were pulsed overnight with 1 μCi of ^{3}H-Thymidine per well before harvesting as described in Figure 2. Specific growth inhibition was calculated from incorporation data of control cultures without peptide added.

Induction of ras specific T cells by primary immunization in vitro.

We first investigated if the T cell repertoire of normal healthy persons contained T cells capable of recognizing and responding to a panel of peptides carrying amino acid sequences derived from mutated p21 ras.

Peripheral blood mononuclear cells from healthy donors were stimulated in vitro with mixtures of synthetic p21 ras peptides according to the present invention as described in Figure 1.

The results of some of these experiments are shown in Table 3. No primary response against the peptide mixtures could be observed. Similar experiments with a panel of 15 healthy donors stimulated with individual peptides also demonstrated a complete lack of primary responses against these peptides

Clone B showed exclusive specificity for peptide 44 which contains the amino acid arginine in position 12. None of the other peptides were recognized (Figure 2b).

As with clone I, clone B was also HLA-DR restricted as evidenced by blocking studies with monoclonal antibodies (Figure 3b).

Clone E showed exclusive specificity for peptide 43 which contains the amino acid valine in position 13. None of the other peptides were recognized (Figure 2c).

Contrary to the clones recognizing peptide 42 and peptide 44 this clone was not blocked by anti HLA-DR monoclonal antibodies, but instead was blocked by a monoclonal antibody recognizing HLA-DQ (Figure 3c). Anti HLA-DP had no effect. These data show that peptide 43 is bound to HLA-DQ in this particular donor.

Clone F showed a different pattern of reactivity. Clone F gave very strong responses against peptide 45 containing alanine in position 12, which was present in the original peptide mixture. It was unreactive to the other peptides in the mixture (Figure 2d), but reacted to a variable degree towards the other peptides (Figure 2e). The reactivity of this clone seemed to be critically dependent on having amino acid glycine in position 13 since substitution of this glycine with aspartic acid or valine totally abrogated the response. Substitution of glycine in position 12 with the basic amino acid lysine or arginine resulted in low or no responses indicating that such substitutions interfere with the T cell receptor binding site. As with clone E, this clone was also HLA-DQ restricted as demonstrated by blocking by monoclonal antibodies (Figure 3d). One interesting observation is that the two peptides carrying basic amino acids in position 12, and which fail to stimulate the HLA-DQ restricted clone F, both are capable of binding to HLA-DR and are

of no importance for the recognition of peptide by clone F. Since peptide 79 lacking the duplicated amino acids was equally efficient in stimulating clone F. Peptides 64, 79 and 80 stimulate clone E in a concentration of 10µl. Peptide 65 and 81 become stimulatory in a concentration of 250µl. Peptides 66, 67, 68, 82 and 83 inhibit at this concentration (Figure 7), and are thus capable of binding to HLA-DQ. Peptides 69, 70, 74, 75 and 76 stimulate clone F at 10µl concentrations.

The combined data in Figure 6-8 show that several peptides carrying Ala in position 12 or Val in position 13 and varying in length, can be recognized by clones E and F. The striking symmetry in the results with clone E and F demonstrate that the amino acids Val in position 8 and Ser in position 17 are absolutely essential for stimulation of these T cell clones. A predicted minimum peptide for stimulation of clone E will therefore have the sequence Val-Val-Gly-Ala-Gly-Val-Val-Gly-Lys-Ser and for Clone F the sequence Val-Val-Gly-Ala-Gly-Val-Val-Gly-Val-Gly-Lys-Ser. The predicted minimum peptide 8-17, 12-Ala,(peptide 88) was synthesized and its capacity to stimulate clone F was compared to peptide 45 and several other peptides as depicted in Figure 16. The dose-response curve for peptide 88 confirms its stimulatory capacity, but shows that it is far less potent than the peptide used for eliciting this clone. Shorter peptides still bind HLA-DQ, but have lost amino acids critical for recognition by these T cell clones. We anticipate that also these peptides, when used to stimulate T cells in vitro, may give rise to a new set of T cell clones with slightly different specificities.

In order to investigate if the same amino acids that were identified as being important for peptide recognition by the HLA-DQ restricted T cell clones were also essential for the recognition by the DR restricted clones B and I, we synthesized truncated forms of the peptides 42 and 44 as shown in Table 2. The peptides were truncated both from the

2. Peptides can bind to both HLA-DR and DQ molecules, since peptides are recognized in a HLA-DR restricted manner (Clone I and B) and in a HLA-DQ restricted manner (clone E and F).
3. By using truncated peptides we have defined putative minimum sequences required for stimulatation of our DQ and DR restricted T cell clones. All clones require the presence of a core sequence spanning positions 8-16 and differ only slightly in the requirements of amino acids in the positions flanking this sequence.
4. Both the DQ and DR restricted clones can respond to peptides of varying length, from the short core sequence up to sequences of 25 amino acids.
5. Our T cell clones have also provided us with important insight into those peptides where no reactive clones are available. Thus, by blocking experiments using 25-50 fold excess of peptides, and the clones E, F, and I as indicator responder cells, we have demonstrated as shown in Figure 5 that all the synthetic peptides in this study, including the peptides representing ras mutations in amino acid 61, are capable of binding to HLA-DQ, and that several of the peptides are capable of binding to HLA-DR.

Having achieved responses in healthy donors by primary immunization in vitro with the shorter peptides, (peptides 42, 43, 44, 45), and observed that T cell clones elicited by stimulation with these peptides were capable of responding to the longer peptides, which presumably required processing, we investigated if the long peptides also would elicit T cell responses after primary stimulation. For these experiments we selected normal donors having HLA molecules not previously known to bind ras peptides. The results from these experiments appear in Figure 19. Peptides 114 and 115 were capable of stimulating specific T cell responses, and T cell clones recognizing these peptides could be generated from bulk cultures. Thus peptides spanning the sequence 1-25 and containing amino acid substitutions in position 12 are also immunogenic. Our results also show that ras peptide

NK) cell activation is responsible for the severe side effects seen in patients given treatment with LAK or TIL cell preparations.

The new finding that the T cell repertoire of a normal individual contain T cells capable of specifically recognizing peptides containing several of the mutations commonly found in ras oncogenes in human cancers, is of importance in cancer therapy and the prevention of cancer by prophylactic vaccination. Furthermore it is also important that all of the synthetic peptides from ras oncogenes are capable of binding to HLA gene products as demonstrated in the peptide blocking experiments.

Of central importance is the finding as shown below, that peripheral blood from a patient with follicular carcinoma of the thyroid gland contain lymphocytes capable of giving a classical memory response against a ras p21 synthetic peptide representing one of the ras mutations commonly found in this cancer form. Such a response is not to be expected unless the patient's T cells have experienced a prior exposure to the same or a very similar peptide fragment <u>in vivo</u>. Such exposure is easily envisaged to occur if the cancer cell of the patient harbour this specific mutation of amino acid 61.

<u>Demonstration of ras peptide specific memory T cells in cancer patients</u>

The synthetic peptides employed for induction of ras peptide specific T cells by primary immunization in vitro, were constructed without precise knowledge of the composition of naturally occurring ras peptides formed by processing of the mutated ras gene product by proteolytic enzymes in vivo.

Although we have demonstrated that each clone shows specifi-

23 to elicit a strong T cell respone in a cancer patient, in a classical memory T cell assay, strongly suggests that the T cells have encountered an identical or very similar peptide in vivo.

In order to further investigate this, we synthesized a series of truncated forms of the peptide 23, and tested their capacity to stimulate clone 15. Data given in Figure 14 show that the N terminal amino acids Asp in position 54 and Ile in position 55 are critical for recognition by clone 15. Removal of Asp 54 strongly reduces the response, and removal of Ile 55 totally abrogates the response. As shown in figure 14, clone 15 was insensitive to the removal of the C terminal amino acids Asp 69, Arg 68, Met 67 and Ala 66. Removal of Ser 65 strongly reduced the response. Together these data indicate that the processed p21 ras peptide which originally stimulated these T cells in vivo may have contained additional amino acids from the N terminal sequence of the ras protein. We accordingly synthesized new peptides that lacked several of the C terminal amino acids that were found not to contribute to T cell recognition, but contained the new amino acids Leu 53, Leu 52 and Cys 51, derived from the natural p21 ras sequence. Data showing stimulation of clone 14 with this set of peptides are given in Figure 15. Optimal stimulation was seen with peptide 106 which encompasses the sequence 51-67. The observation that an even stronger response is achieved with a peptide having a sequence which differs significantly from the peptide used for in vitro stimulation of the T cell giving rise to the clone, strongly indicates that peptide 106 is more representative of the peptide processed by the cancer cell and originally giving rise to an immune response than peptide 23.

It is noteworthy that the cancer type of the patient is the only where high incidences of ras mutations in position 61 have been reported.

coupling. Detachment from the resin and final removal of side chain protection was performed by 95% TFA (aq.). The peptides were purified and analysed by reversed phase (C18) HPLC (Shimadzu LC8A). Amino acid analysis was carried out using the PICO-Tag method (Waters Millipore Inc.).

The following peptides and peptide fragments were synthesized by this method:

A1). ras peptides of the following sequence having mutation points in position 12 or 13:

6 7 8 9 10 11 12 13 14 15 16 17 18 19
Leu-Val-Val-Val-Gly-Ala-Gly-**Val**-Val-Gly-Lys-Ser-Ala-Leu

Other synthesized peptides having the sequence 1-25 of the normal ras peptide, but carrying mutations in position 12 or 13 will appear from Table 8.

A2) ras peptides of the following sequence having mutation points in position 12 or 13 and additional amino acids at one end not belonging to the natural sequence of the ras proteins:

6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
Leu-Val-Val-Val-Gly-Ala-**Gly-Gly**-Val-Gly-Lys-Ser-Ala-Leu-Ala-Leu
Leu-Val-Val-Val-Gly-Ala-**Asp**-Gly-Val-Gly-Lys-Ser-Ala-Leu-Ala-Leu
Leu-Val-Val-Val-Gly-Ala-**Val**-Gly-Val-Gly-Lys-Ser-Ala-Leu-Ala-Leu
Leu-Val-Val-Val-Gly-Ala-**Cys**-Gly-Val-Gly-Lys-Ser-Ala-Leu-Ala-Leu
Leu-Val-Val-Val-Gly-Ala-**Ser**-Gly-Val-Gly-Lys-Ser-Ala-Leu-Ala-Leu
Leu-Val-Val-Val-Gly-Ala-**Lys**-Gly-Val-Gly-Lys-Ser-Ala-Leu-Ala-Leu
Leu-Val-Val-Val-Gly-Ala-**Arg**-Gly-Val-Gly-Lys-Ser-Ala-Leu-Ala-Leu
Leu-Val-Val-Val-Gly-Ala-**Ala**-Gly-Val-Gly-Lys-Ser-Ala-Leu-Ala-Leu
Leu-Val-Val-Val-Gly-Ala-Gly-**Val**-Val-Gly-Lys-Ser-Ala-Leu-Ala-Leu
Leu-Val-Val-Val-Gly-Ala-Gly-**Asp**-Val-Gly-Lys-Ser-Ala-Leu-Ala-Leu

C). abl-bcr fusion gene peptide:
Ile-Pro-Leu-Thr-Ile-Asn-Lys-Glu-Glu-Ala-Leu-Gln-
Arg-Pro-Val-Ala-Ser-Asp-Phe-Glu

Ala-Thr-Gly-Phe-Lys-Gln-Ser-Ser-Lys-Ala-Leu-Gln-
Arg-Pro-Val-Ala-Ser-Asp-Phe-Glu

d). egf receptor peptide and retinoid receptor peptide
Ser-Arg-Ala-Leu-Glu-Glu-Lys-Lys-Gly-Asn-Tyr-Val-
Val-Thr-Asp-His-Gly

Leu-Ser-Ser-Cys-Ile-Thr-Gln-Gly-Lys-Ala-Ile-Glu-
Thr-Gln-Ser-Ser-Ser-Ser-Glu-Glu

TRUNCATED p21 ras PEPTIDES USED ...

p75	Leu-Val-Val-Val-Gly-Ala-Ala-Gly-Val-Gly-Lys-Ser-Ala-Leu-Ala-Leu
p74	Leu-Val-Val-Val-Gly-Ala-Ala-Gly-Val-Gly-Lys-Ser-Ala-Leu
p75	Leu-Val-Val-Val-Gly-Ala-Ala-Gly-Val-Gly-Lys-Ser-Ala
p76	Leu-Val-Val-Val-Gly-Ala-Ala-Gly-Val-Gly-Lys-Ser
p77	Leu-Val-Val-Val-Gly-Ala-Ala-Gly-Val-Gly-Lys
p78	Leu-Val-Val-Val-Gly-Ala-Ala-Gly-Val-Gly
p69	-Val-Val-Val-Gly-Ala-Ala-Gly-Val-Gly-Lys-Ser-Ala-Leu-Ala-Leu
p70	-Val-Val-Gly-Ala-Ala-Gly-Val-Gly-Lys-Ser-Ala-Leu-Ala-Leu
p71	-Val-Gly-Ala-Ala-Gly-Val-Gly-Lys-Ser-Ala-Leu-Ala-Leu
p72	-Gly-Ala-Ala-Gly-Val-Gly-Lys-Ser-Ala-Leu-Ala-Leu
p73	-Ala-Ala-Gly-Val-Gly-Lys-Ser-Ala-Leu-Ala-Leu
p88	Val-Val-Gly-Ala-Gly-Val-Gly-Lys-Ser
p89	Val-Val-Val-Gly-Ala-Gly-Val-Gly-Lys-Ser
p90	Val-Val-Gly-Ala-Gly-Val-Gly-Lys-Ser-Ala
p91	Val-Val-Val-Gly-Ala-Gly-Val-Gly-Lys-Ser-Ala

Table 2

p177	Leu-Val-Val-Val-Gly-Ala-Arg-Gly-Val-Gly
p178	Leu-Val-Val-Val-Gly-Ala-Arg-Gly-Val
p179	Leu-Val-Val-Val-Gly-Ala-Lys-Gly-Val-Gly
p180	Leu-Val-Val-Val-Gly-Ala-Lys-Gly-Val
p181	Val-Gly-Ala-Lys-Gly-Val-Gly-Lys-Ser-Ala-Leu-Ala-Leu
p182	Gly-Ala-Lys-Gly-Val-Gly-Lys-Ser-Ala-Leu-Ala-Leu

Table 2 cont.

The specificity of the clones:

16 out of 45 clones show specificity for the

peptide mixture or a single peptide.

Peptide 42 2/45

Peptide 43 9/45

Peptide 44 1/45

Peptide 45 1/45

Peptide 46 0/45

Peptide mixture: 3/45

Table 6

THE SEQUENCE OF THE AMINO-ACIDS FROM POSITION 1 TO 26 IN THE p21 h-, k- AND N-RAS PROTEIN:

Met-Thr-Glu-Tyr-Lys-Leu-Val-Val-Val-Gly-Ala-Gly-Gly-Val-Gly-Lys-Ser-Ala-Leu-Thr-Ile-Gln-Leu-Ile-Gln-Asn

THE SEQUENCE OF THE AMINO-ACIDS FROM POSITION 45 TO 72 IN THE p21 H-, K- AND N-RAS PROTEIN:

Val-Ile-Asp-Gly-Glu-Thr-Cys-Leu-Leu-Asp-Ile-Leu-Asp-Thr-Als-Gly-Gln-Glu-Glu-Tyr-Ser-Ala-Met-Arg-Asp-Gln-Tyr-Met

THE SEQUENCE OF THE AMINO-ACIDS FROM POSITION 53 TO 67 IN THE p21 R-RAS PROTEIN:

Leu-Asp-Ile-Leu-Asp-Thr-Ala-Gly-Gln-Glu-Glu-Phe-Gly-Ala-Met

Table 8

P 112:
1 2 3 4 5 6 7 8 9 10 11
Met-Thr-Glu-Tyr-Lys-Leu-Val-Val-Val-Gly-Ala-
12 13 14 15 16 17 18 19 20 21 22
Gly-Gly-Val-Gly-Lys-Ser-Ala-Leu-Thr-Ile-Gln-
23 24 25
Leu-Ile-Gln

P 113:
1 2 3 4 5 6 7 8 9 10 11
Met-Thr-Glu-Tyr-Lys-Leu-Val-Val-Val-Gly-Ala-
12 13 14 15 16 17 18 19 20 21 22
Val-Gly-Val-Gly-Lys-Ser-Ala-Leu-Thr-Ile-Gln-
23 24 25
Leu-Ile-Gln

P 114:
1 2 3 4 5 6 7 8 9 10 11
Met-Thr-Glu-Tyr-Lys-Leu-Val-Val-Val-Gly-Ala-
12 13 14 15 16 17 18 19 20 21 22
Lys-Gly-Val-Gly-Lys-Ser-Ala-Leu-Thr-Ile-Gln-
23 24 25
Leu-Ile-Gln

P 115:
1 2 3 4 5 6 7 8 9 10 11
Met-Thr-Glu-Tyr-Lys-Leu-Val-Val-Val-Gly-Ala-
12 13 14 15 16 17 18 19 20 21 22
Arg-Gly-Val-Gly-Lys-Ser-Ala-Leu-Thr-Ile-Gln-
23 24 25
Leu-Ile-Gln

P 116:
1 2 3 4 5 6 7 8 9 10 11
Met-Thr-Glu-Tyr-Lys-Leu-Val-Val-Val-Gly-Ala-
12 13 14 15 16 17 18 19 20 21 22
Ala-Gly-Val-Gly-Lys-Ser-Ala-Leu-Thr-Ile-Gln-
23 24 25
Leu-Ile-Gln

P 117:
1 2 3 4 5 6 7 8 9 10 11
Met-Thr-Glu-Tyr-Lys-Leu-Val-Val-Val-Gly-Ala-
12 13 14 15 16 17 18 19 20 21 22
Ser-Gly-Val-Gly-Lys-Ser-Ala-Leu-Thr-Ile-Gln-
23 24 25
Leu-Ile-Gln

Table 9

p158	Val-Ile-Asp-Gly-Glu-Thr-Cys-Leu-Leu-Asp-Ile-Leu-Asp-Thr-Ala-Gly-Leu-Glu-Glu-Tyr-Ser-Ala-Met-Arg-Asp-Gln-Tyr-Met
p166	Val-Ile-Asp-Gly-Glu-Thr-Cys-Leu-Leu-Asp-Ile-Leu-Asp-Thr-Ala-Gly-Arg-Glu-Glu-Tyr-Ser-Ala-Met-Arg-Asp-Gln-Tyr-Met
p168	Val-Ile-Asp-Gly-Glu-Thr-Cys-Leu-Leu-Asp-Ile-Leu-Asp-Thr-Ala-Gly-Lys-Glu-Glu-Tyr-Ser-Ala-Met-Arg-Asp-Gln-Tyr-Met
p167	Val-Ile-Asp-Gly-Glu-Thr-Cys-Leu-Leu-Asp-Ile-Leu-Asp-Thr-Ala-Gly-His-Glu-Glu-Tyr-Ser-Ala-Met-Arg-Asp-Gln-Tyr-Met

BINDING OF PEPTIDES TO AFFINITY PURIFIED HLA MOLECULES

Peptides	Sequences	Results DQw6 IC50 (μM)	DR1 IC50 (μM)
P112	MTEYKLVVVGAGGVGKSALTIQLIQ	6,02	1,21
P113	----------V----------	8,97	0,78
P114	----------K----------	2,53	0,55
P115	----------R----------	2,78	0,24
P116	----------A----------	2,68	0,44
P117	----------S----------	1,91	<0,11
P118	----------C----------	10	1,75
P119	----------D----------	3,03	1,10
P120	----------GV----------	7,31	0,74
P121	----------D----------	2,74	0,31
P88	VVGAAGVGKS	8,97	
P89	V----------	10	
P90	----------A	9,98	
P91	----------	1,29	
P45	L----------LAL	0,90	
P104	Y----------	<0,33	0,78
P34	SGPLKAEIAQLEY	>>10	0,15

Table 11
The binding of peptides to affinity purified HLA molecules was tested by their capasity to inhibit the binding of a radio-labeled indicator peptid. To test the binding of peptides to DQw6, iodinated P104 was used as an indicator peptide. Whereas iodinated P34(derived from Influenza matrix protein aa 17-29) was used as indicator peptide to test the binding of peptides to DR1. In the table the concentration at 50 % inhibition (IC50) of the binding of the indicator peptide is shown. Low IC50 means good binding capasity. The peptides were tested in the concentration range from 0,33 - 10 μM.

Table 11

4. A Pharmaceutical composition comprising a peptide according to any of the claims 1-3 and a pharmaceutically acceptable carrier or diluent.

5. A Cancer Vaccine comprising a peptide according to any of the claims 1-3 and a pharmaceutically acceptable carrier or diluent.

6. Use of a peptide according to any of the claims 1-3 for the preparation of a pharmaceutical composition for eliciting T-cell immunity against tumours.

7. Method for vaccination of a person disposed for cancer consisting of induction of T-cell immunity to oncogene proteins by stimulating in vivo with peptides according to the claims 1-3.

8. Method for treatment of a patient afflicted with cancer consisting of induction of T-cell immunity to oncogene proteins by stimulating in vivo or in vitro with peptides according to the claims 1-3.

Protocol: 1/33

2 mill.cells / ml x 10 ml

20 micrograms of five p21 ras peptides

1 week

restimulation: 1 mill.irrad.autologous feeder cells/ml

and 20 micrograms of each peptide final dilution

1 week

restimulation (as described above)

1 week

cloning: 20 Terasaki plates, 1 blast per well

clones in 51 out of 600 wells

Probability of clonality: 97,8 %

45 clones expanded

29 clones characterized

Figure 1

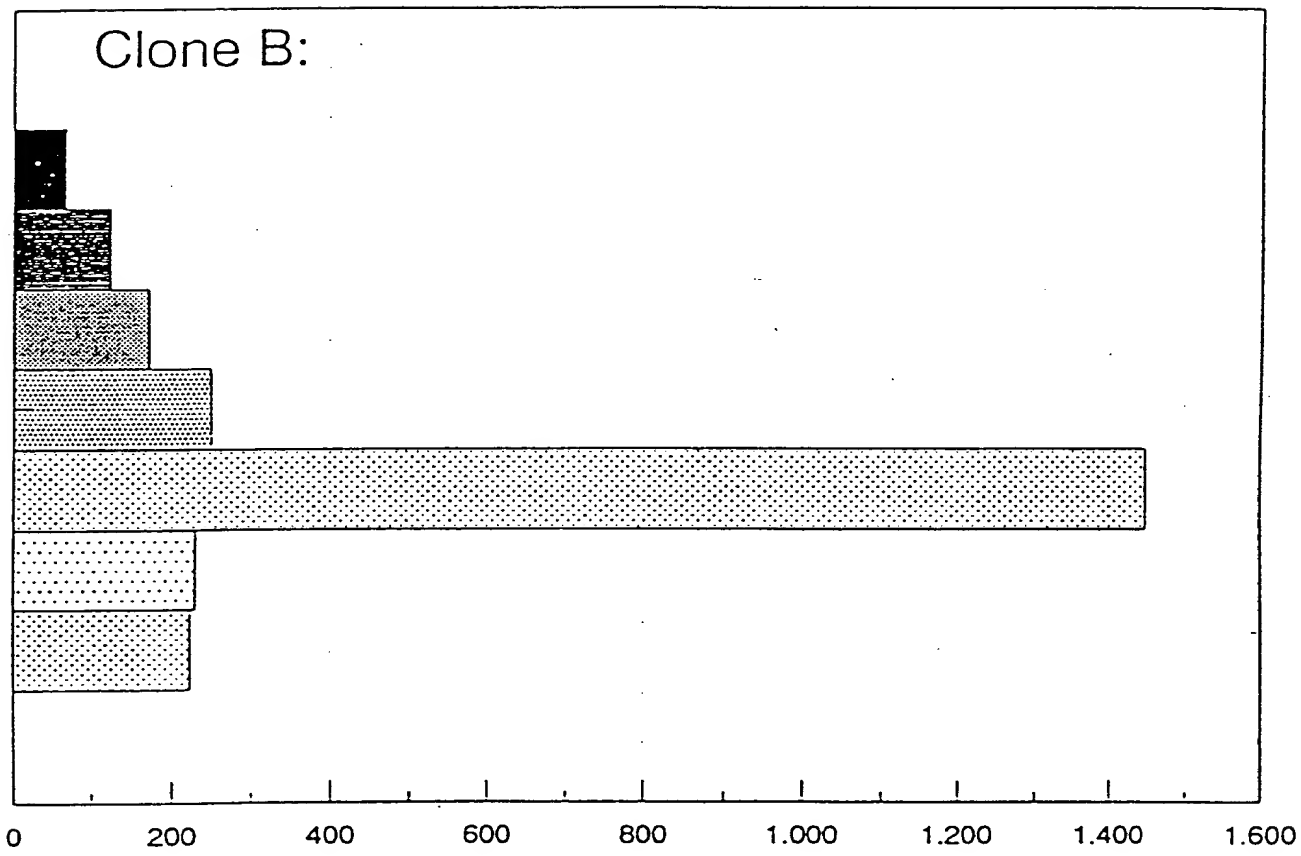

Clone B:
0
200
400
600
800
1.000
1.200
1.400
1.600
cpm
Clone:
Clone+APC:
Clone+APC+P-44:
Clone+APC+P-45:
Clone+APC+P-42:
Clone+APC+P-43:
Clone+APC+P-46:

Figure 2b

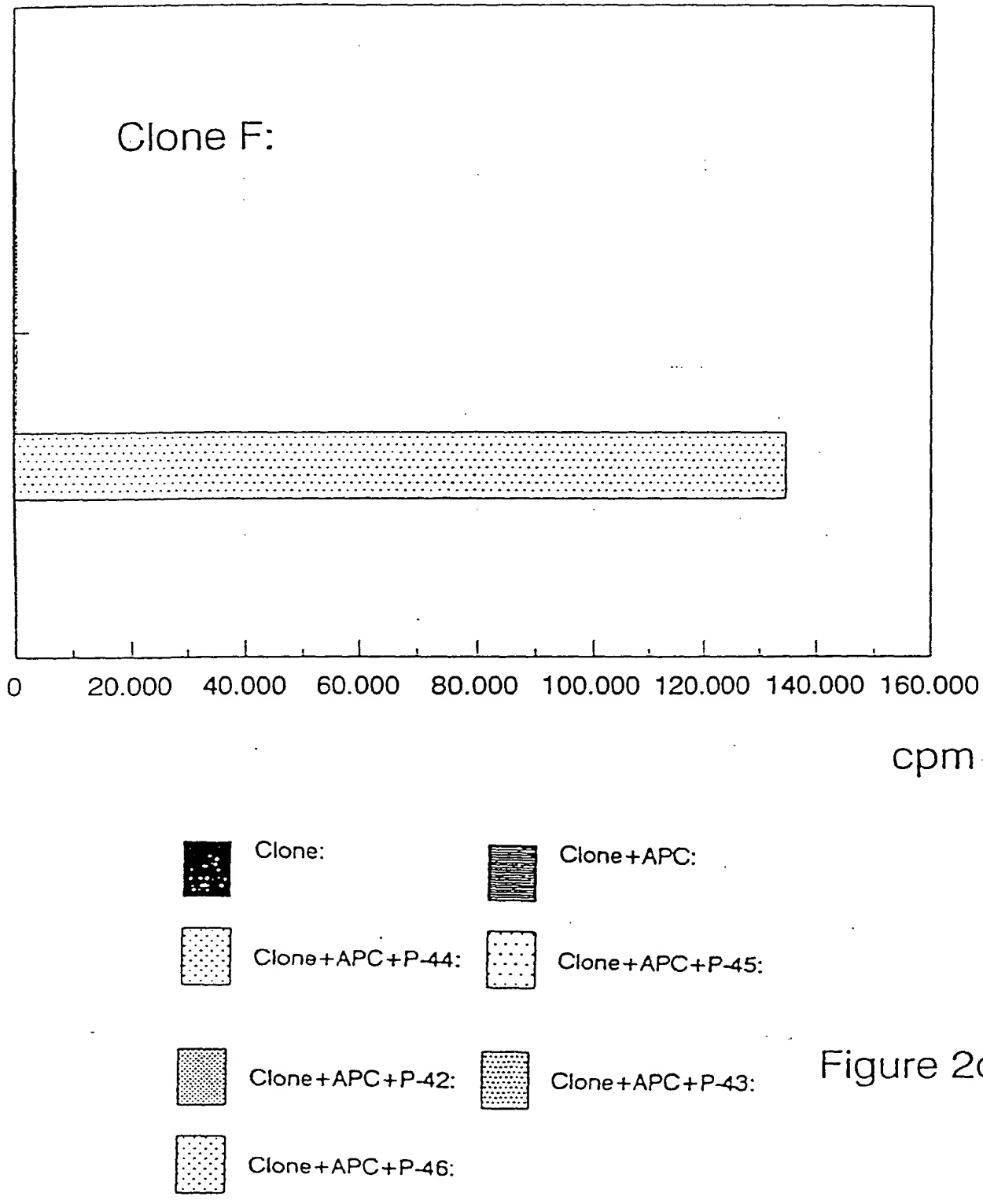
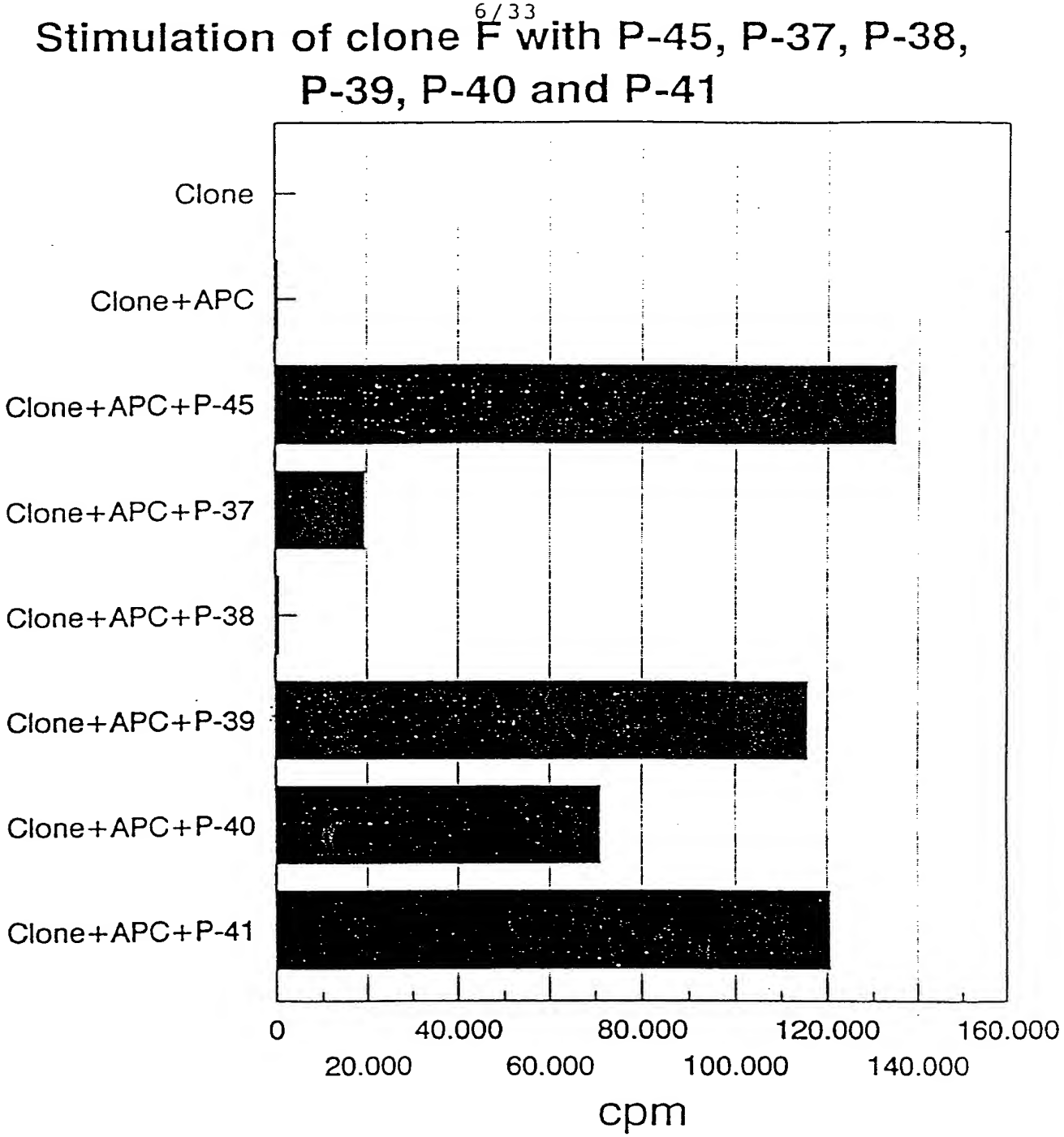

Clone F:
0
20.000
40.000
60.000
80.000
100.000
120.000
140.000
160.000
cpm
Clone:
Clone+APC:
Clone+APC+P-44:
Clone+APC+P-45:
Clone+APC+P-42:
Clone+APC+P-43:
Clone+APC+P-46:

Figure 2d



Clone: 50000 cells/well
APC: 50000 cells/well
Peptideconcentration: 20 micrograms/ml

Figure 2e

Clone I:
Inhibition with the mAb L 243 specific for HLA-DR

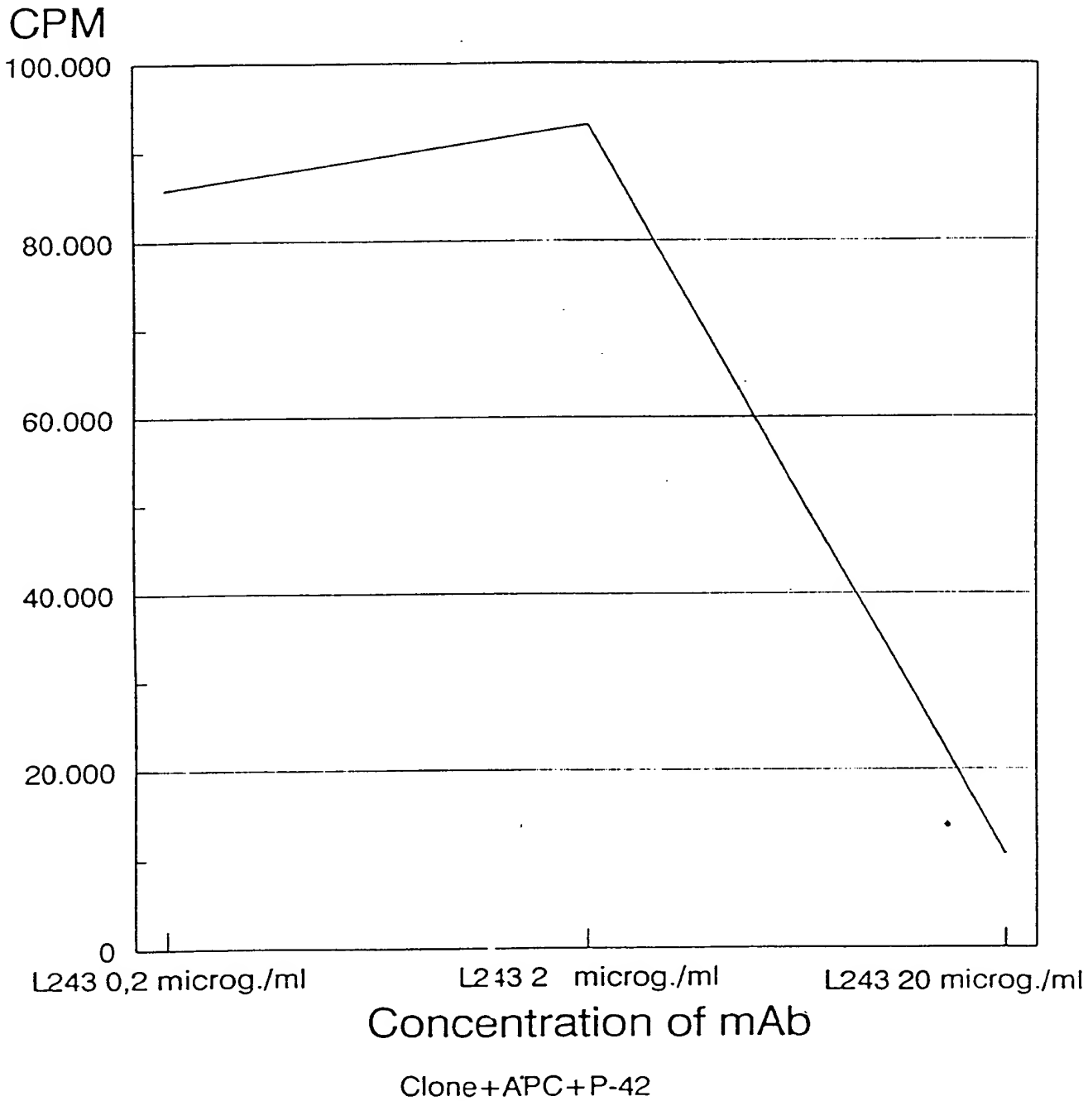


Figure 3a

Clone B:
Inhibition with the mAb L 243 specific for HLA-DR

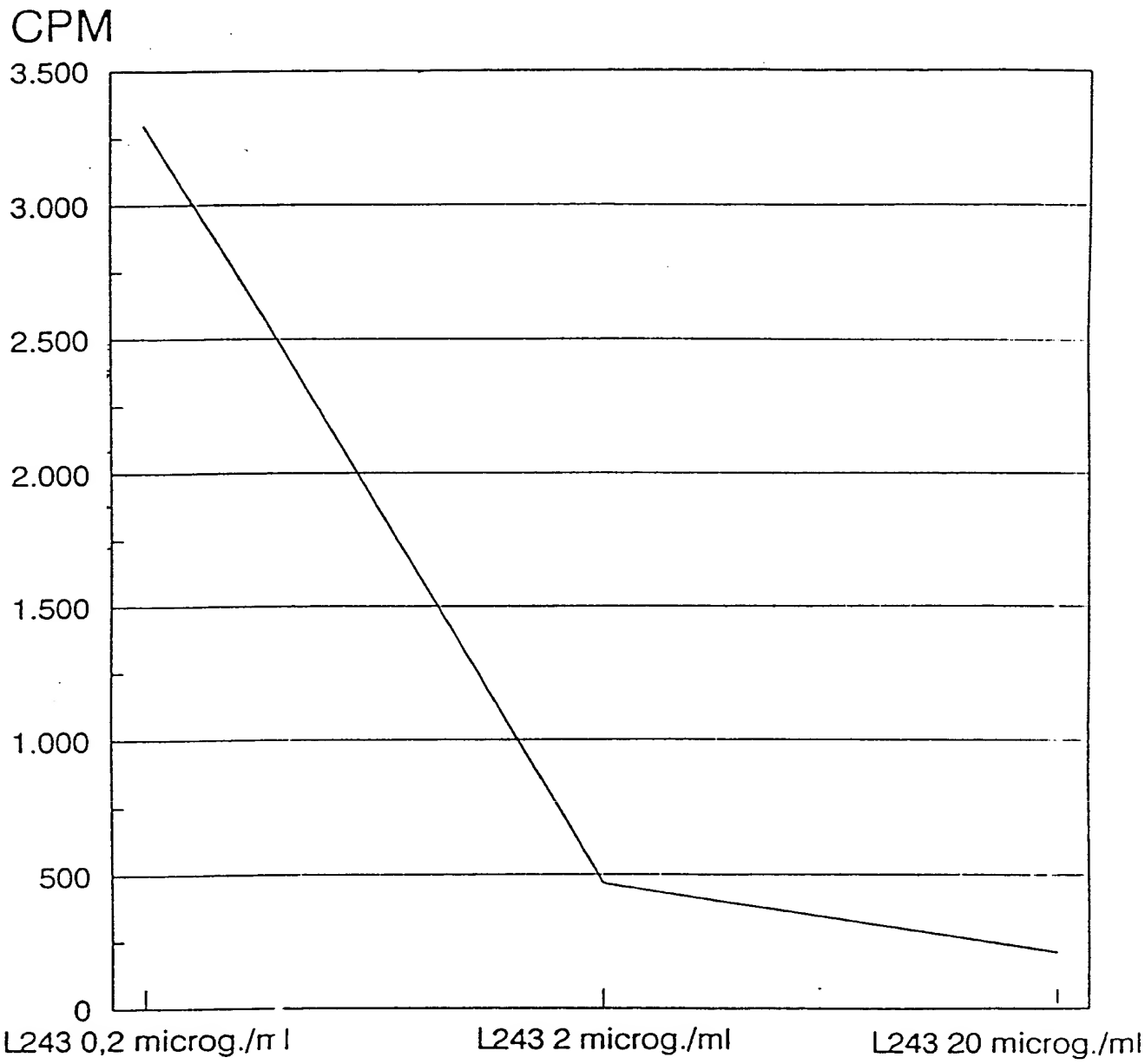


Figure 3b

Clone E:
Inhibition with the mAb FN.81.1.1. specific for HLA-DQ

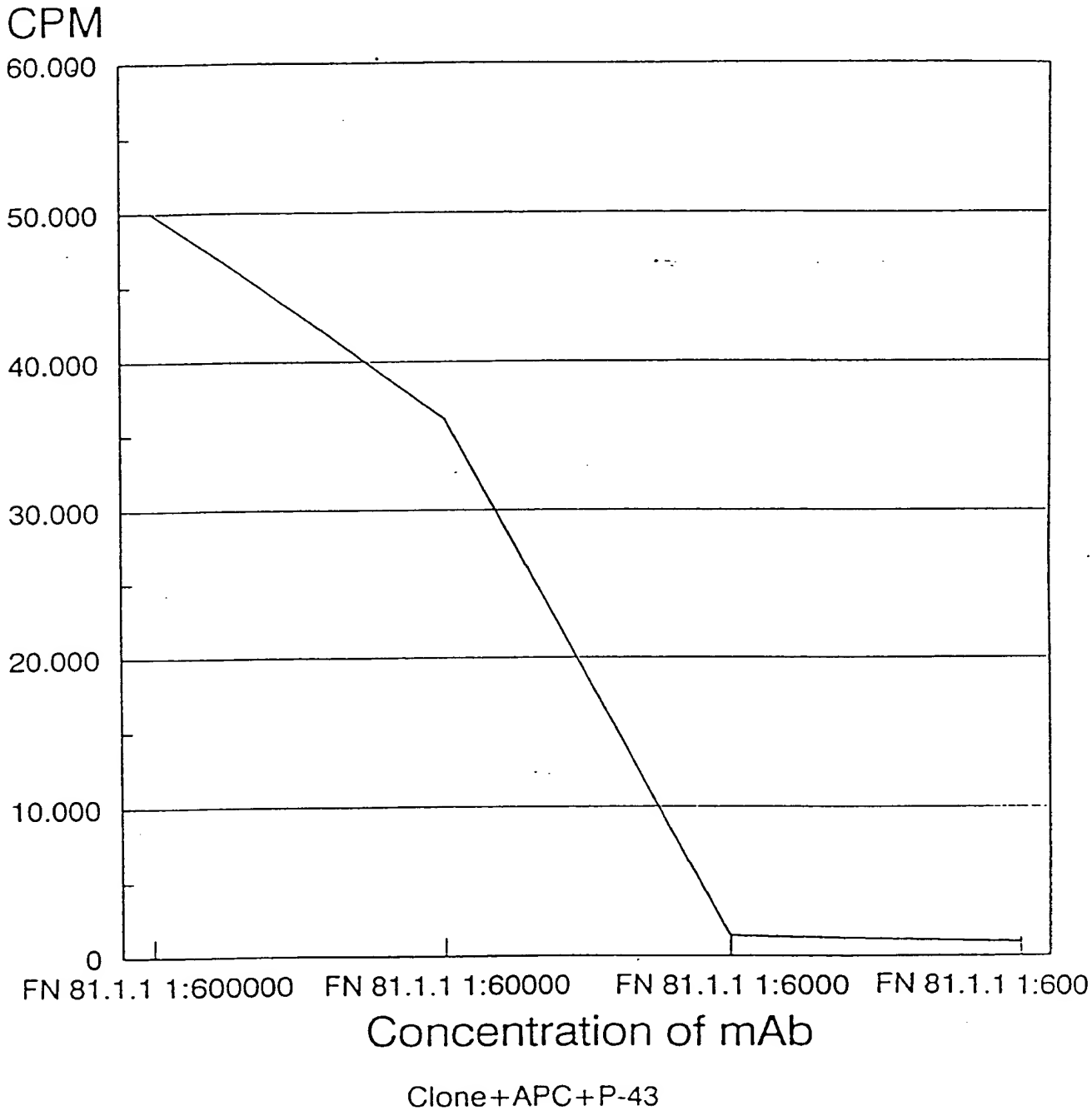


Figure 3c

Clone F:
Inhibition with the Moab FN 81.1.1 specific for HLA-DQ

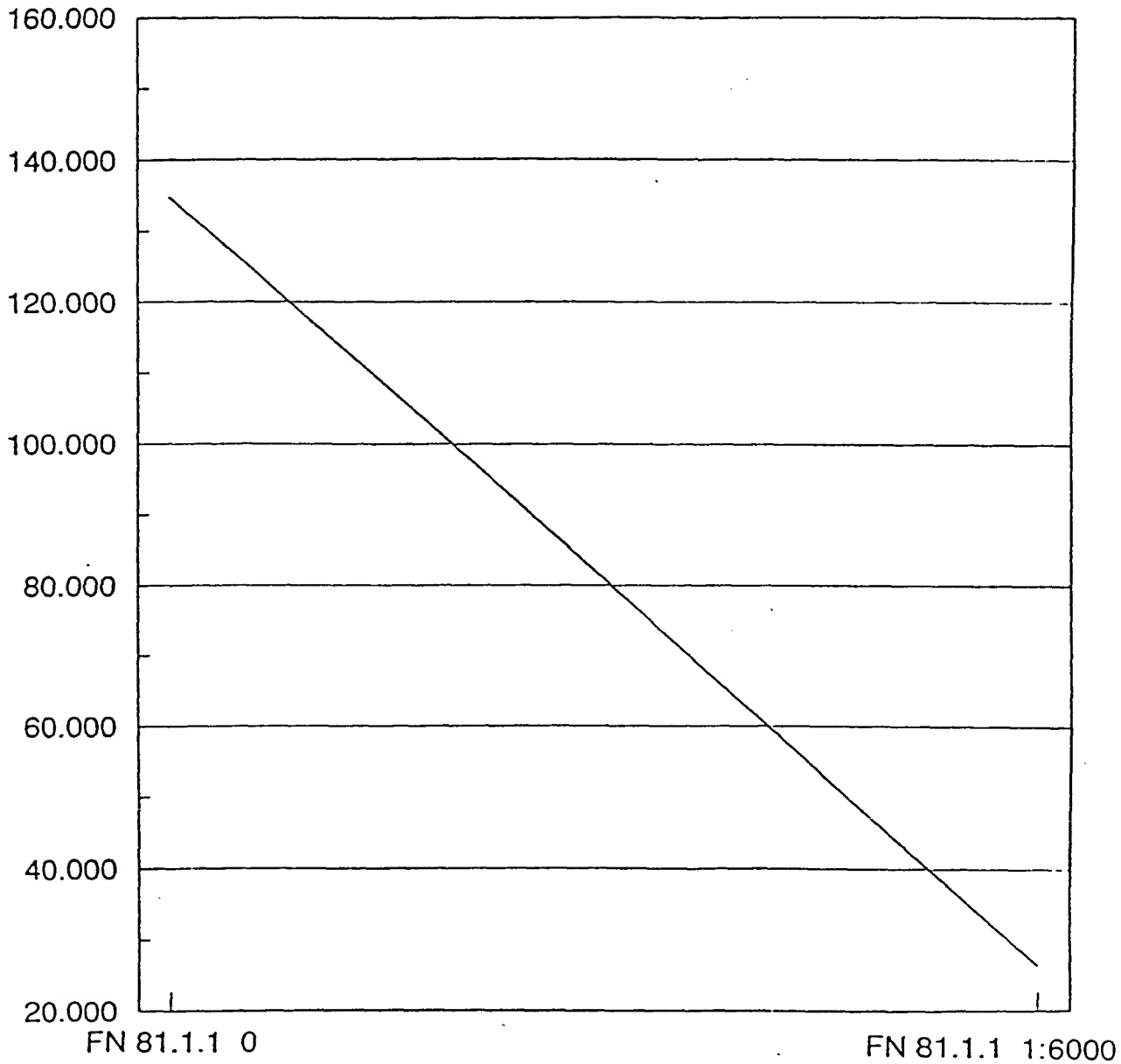
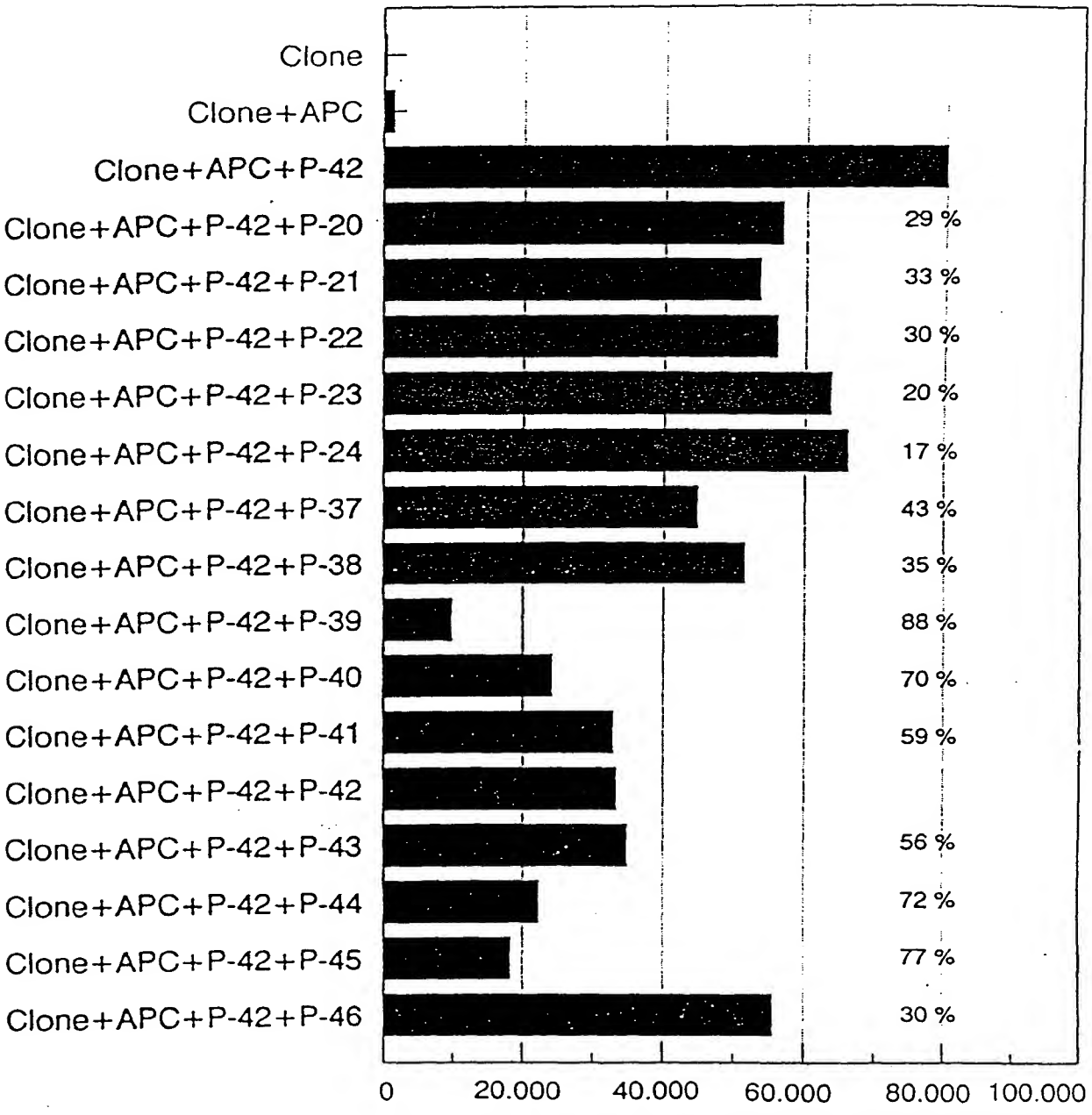


Figure 3d

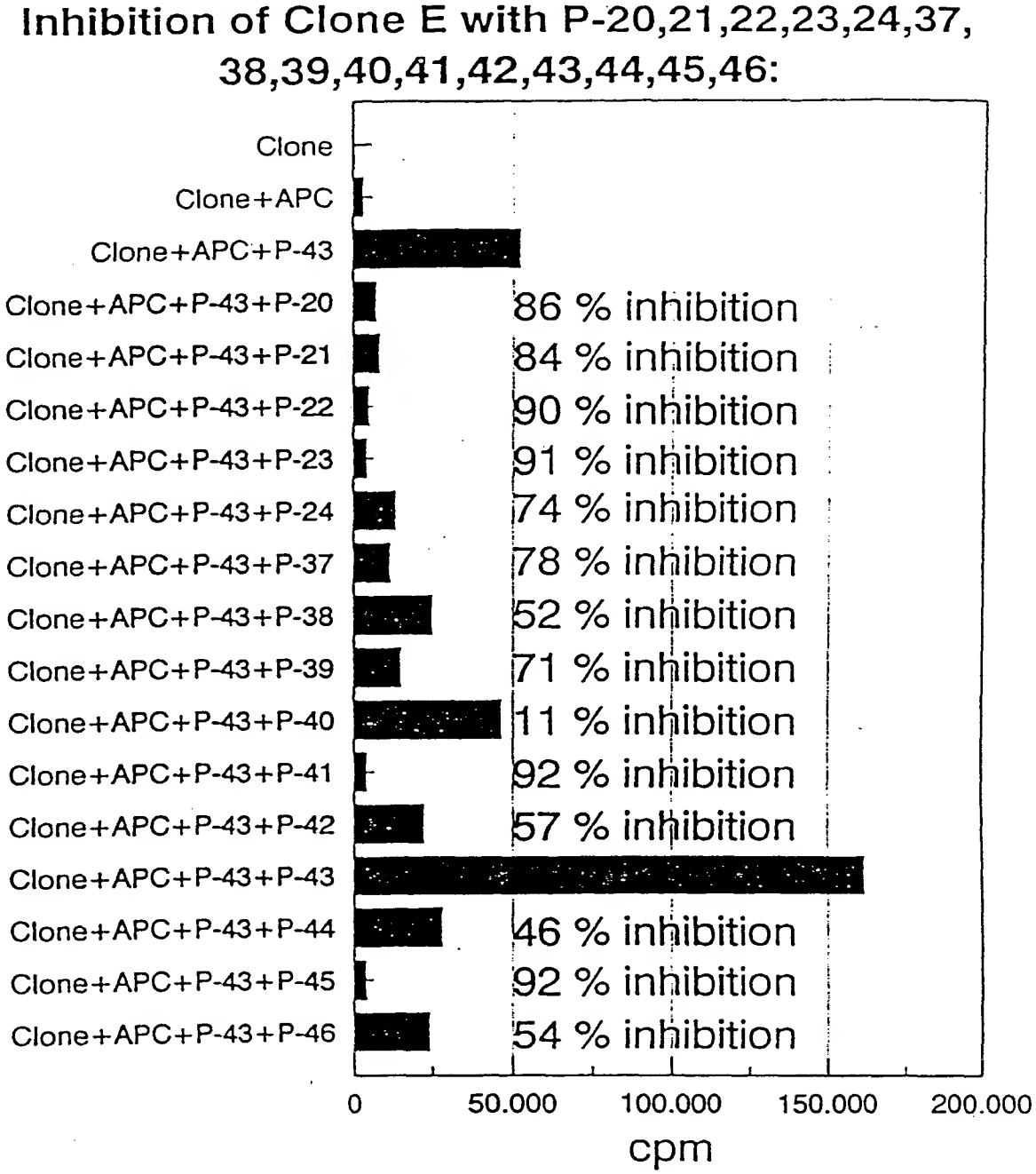
Inhibition of Clone I with P-20,21,22,23,24,37, 38,39,40,41,42,43,44,45,46:



The percentages refer to % inhibition

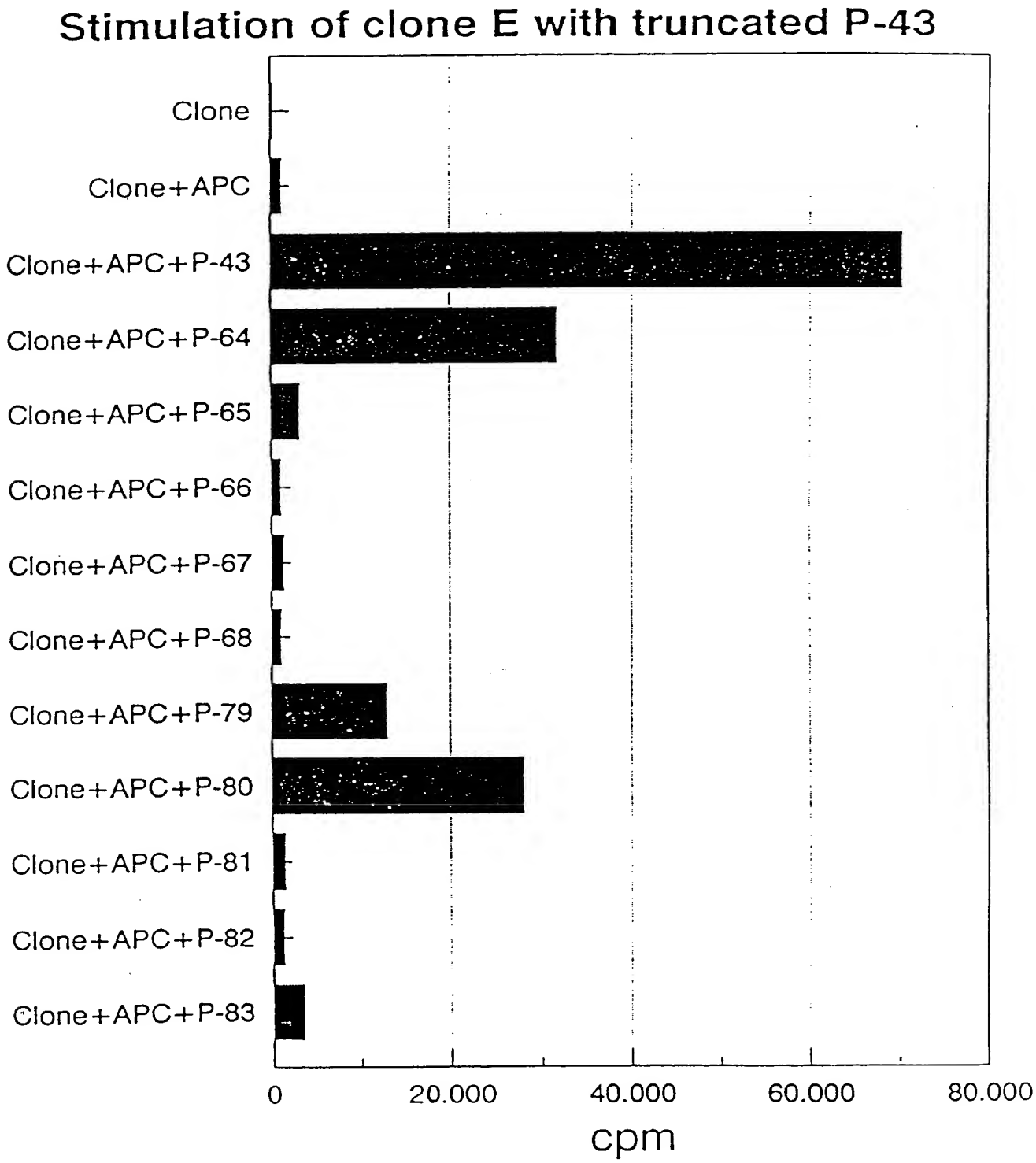
Concentration of inhibiting peptide: 250 micro M
Concentration of stimulating peptide: 10 micro M
Clone: 50000 cells/well, APC: 50000 cells/well

Figure 4



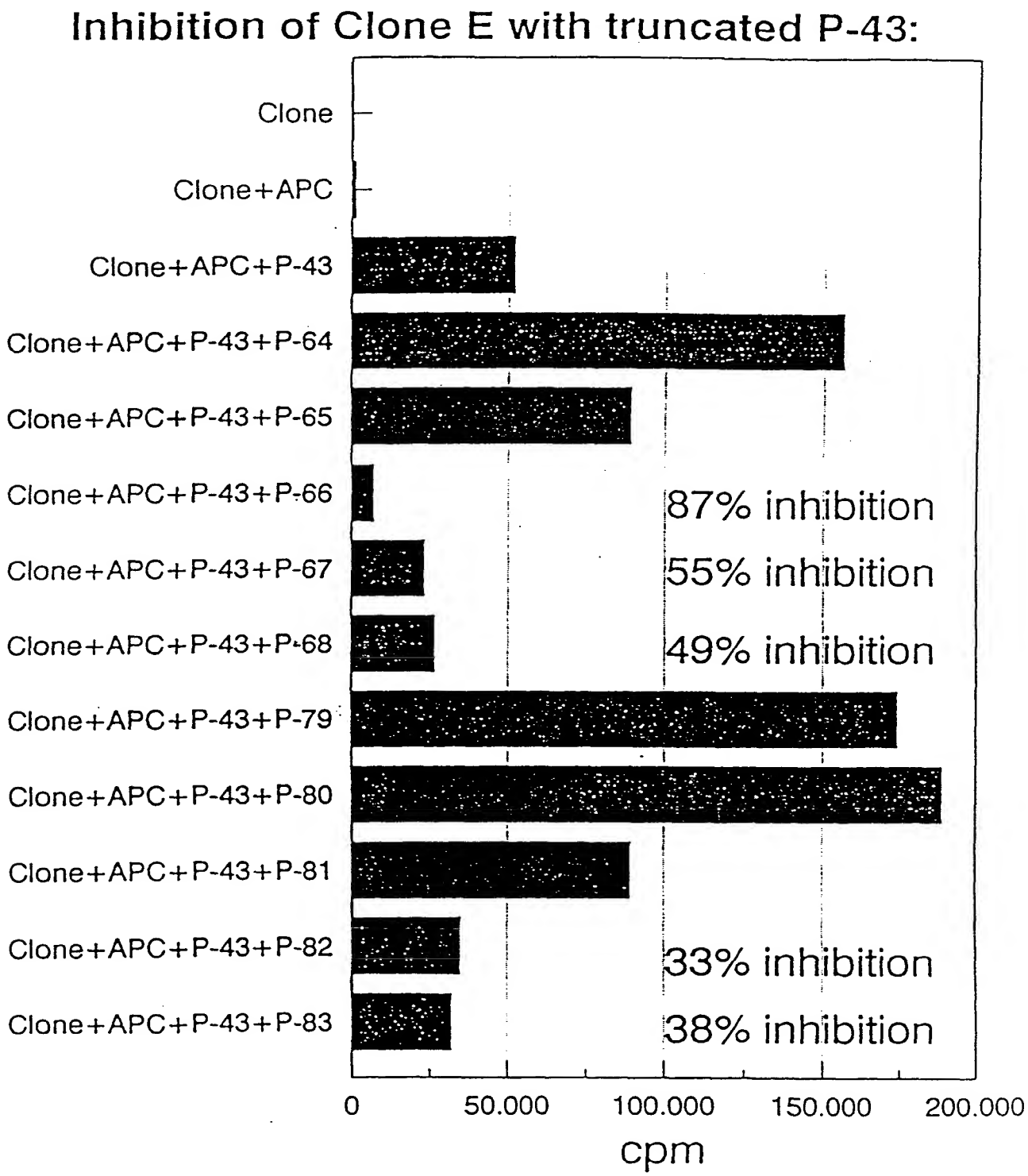
Concentration of inhibiting peptide: 250 micro M
Concentration of stimulating peptide: 10 micro M
Clone: 50000 cells/well, APC: 50000 cells/well

Figure 5



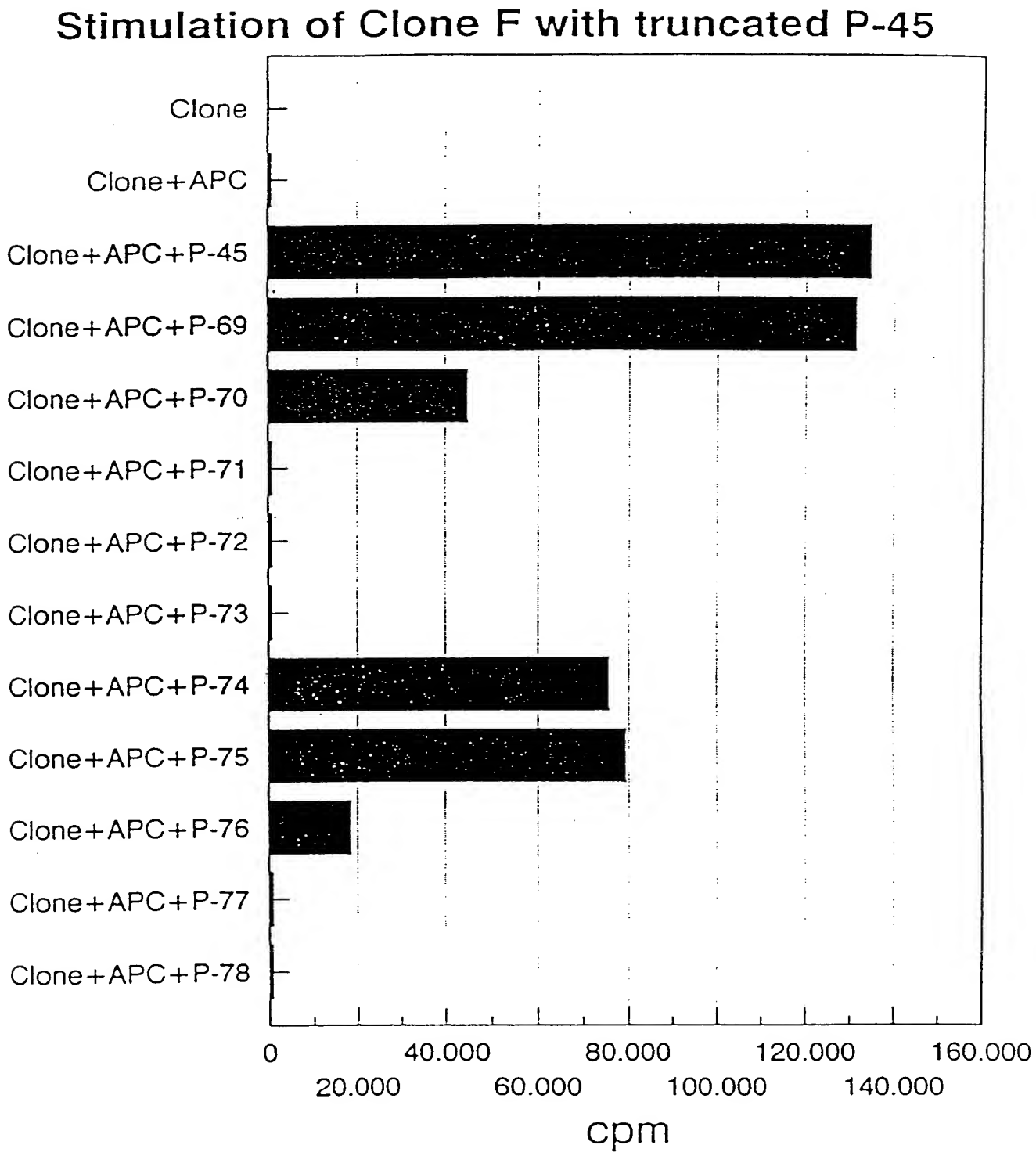
Concentration of stimulating peptide: 10 micro M
Clone: 50 000 cells/well
APC: 50000 cells/well

Figure 6



Concentration of inh biting peptide 250 micro M
Concentration of stimulating peptide 5 micro M
Clone: 50000 cells/well, APC: 50000 cells/well

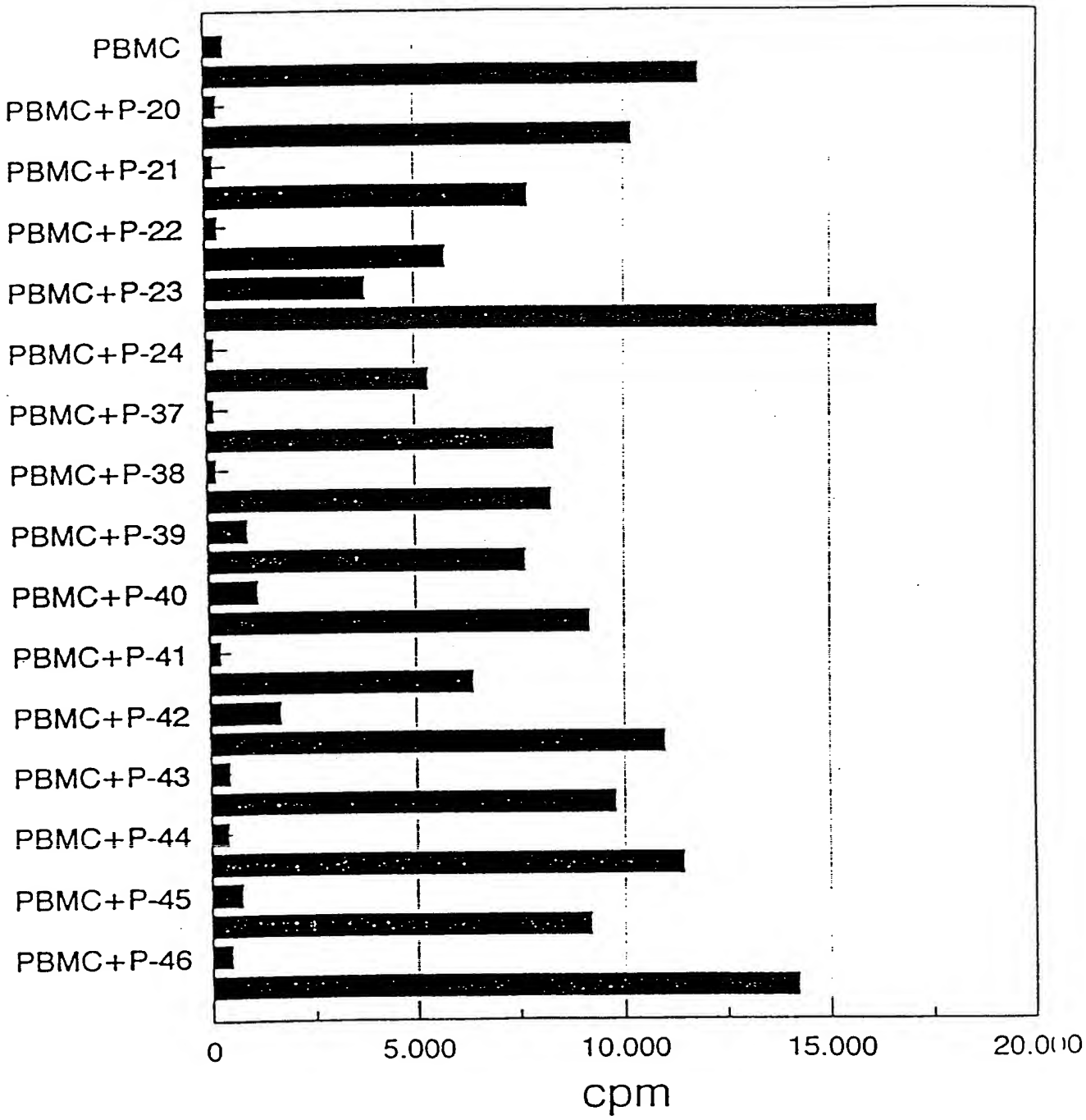
Figure 7



Clone: 50000 cells/well
APC: 50000 cells/well
Concentration of stimulating peptide: 10 micro M

Figure 8

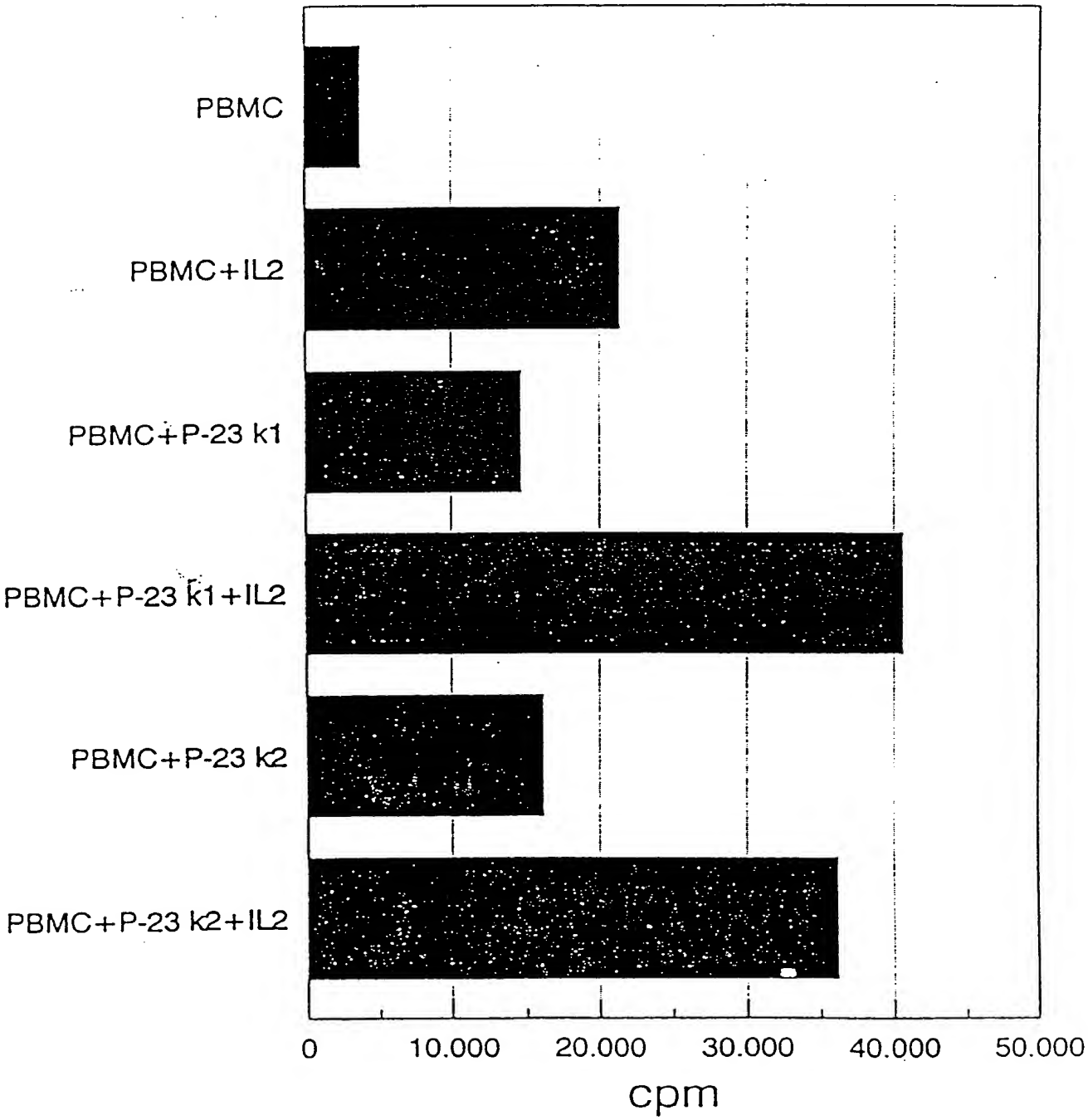
Patient nr.29 Follicular thyroid carcinoma:



PBMC: 100000 cells/well
Peptide concentration: 100 micrograms/ml
IL2 = 1 U/ml

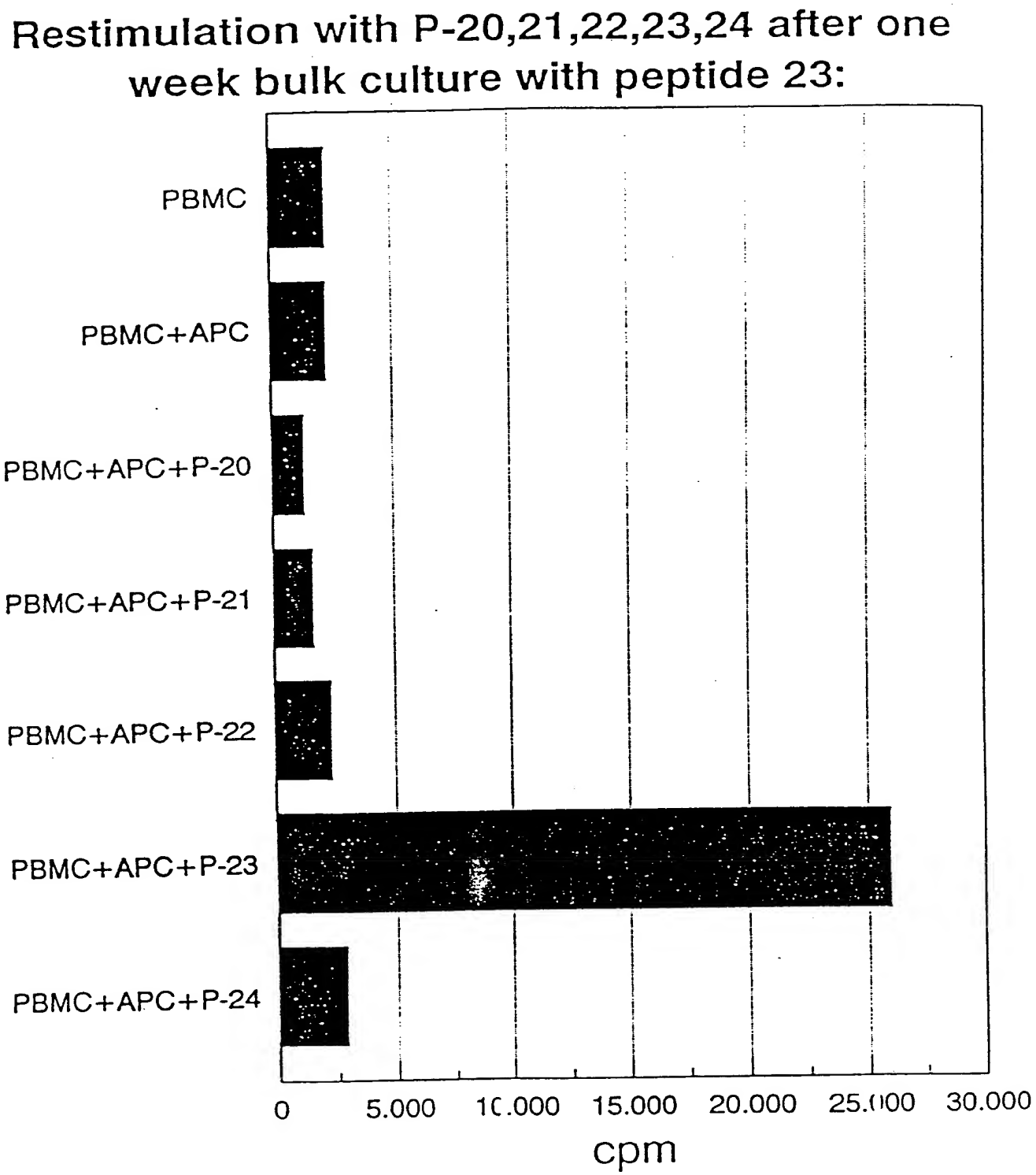
Figure 9

Patient nr.29 Follicular thyroid carcinoma:



PBMC: 200000 cells/well
P-23 k1 = 20 microg./ml, P-23 k2 =200 microg./ml
IL2: 1 U/ml

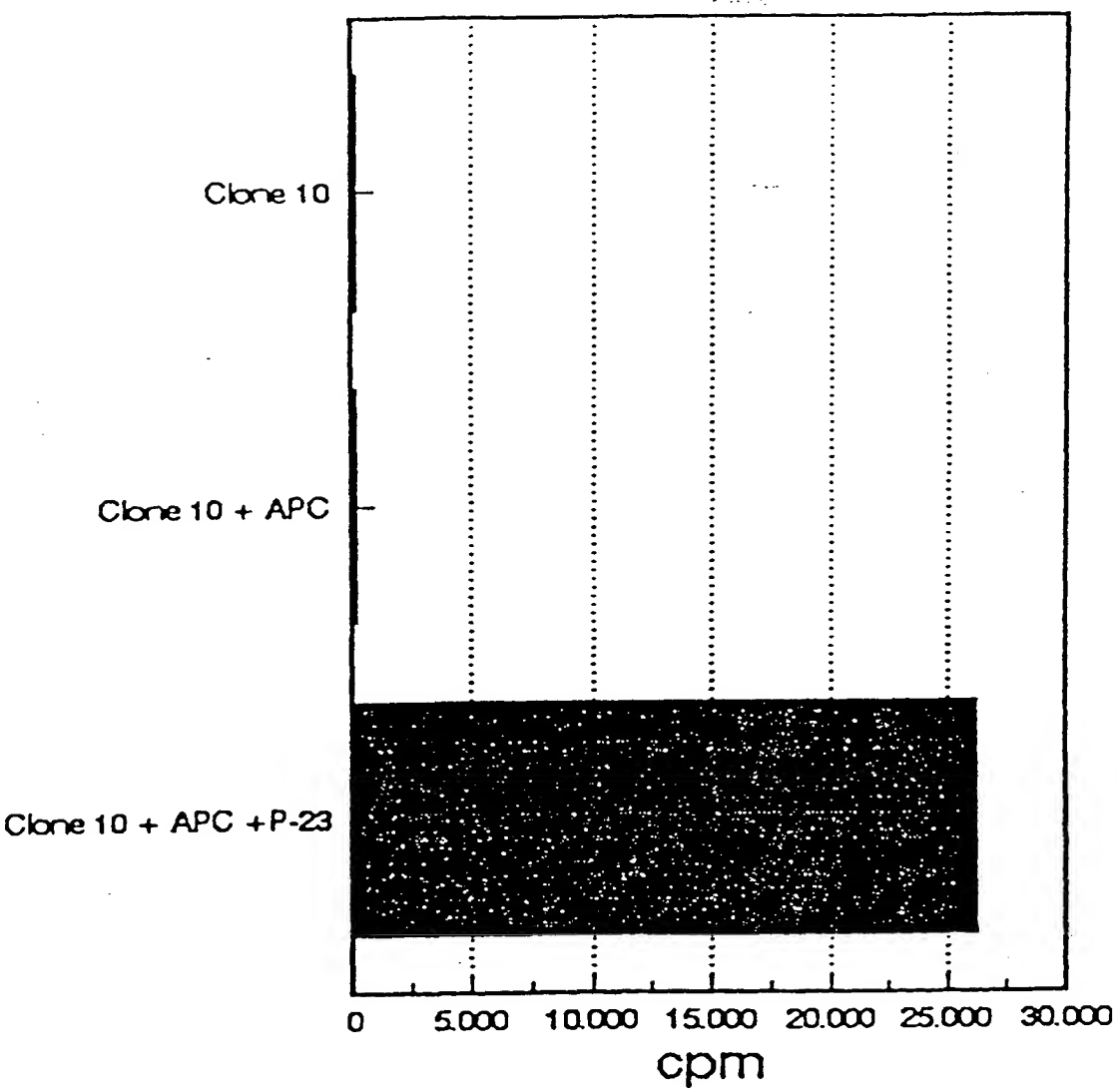
Figure 10



PBMC: 25000 cells/well
APC: 25000 cells/well
Peptide concentration: 200 microg./ml

Figure 11

Peptide 23 specific clones derived from patient nr.29

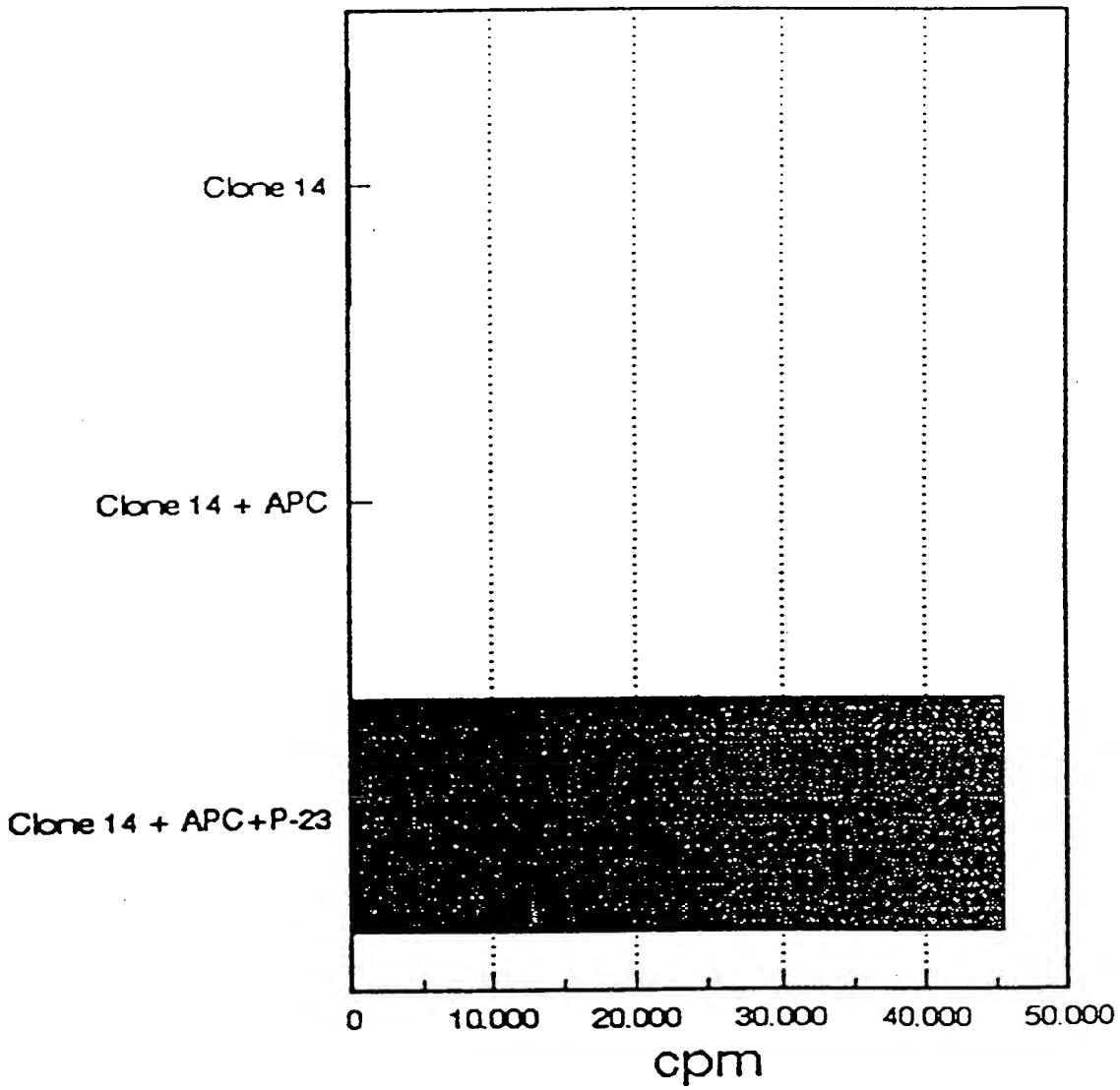


Clone: 50000 cells/well
APC: irradiated allogeneic HLA-DQ identical PBMC 50000 cells/well
Peptide 23: 50 micrograms/ml

Figure 12

Peptide 23 specific clones derived from patient nr.29

(continued...)

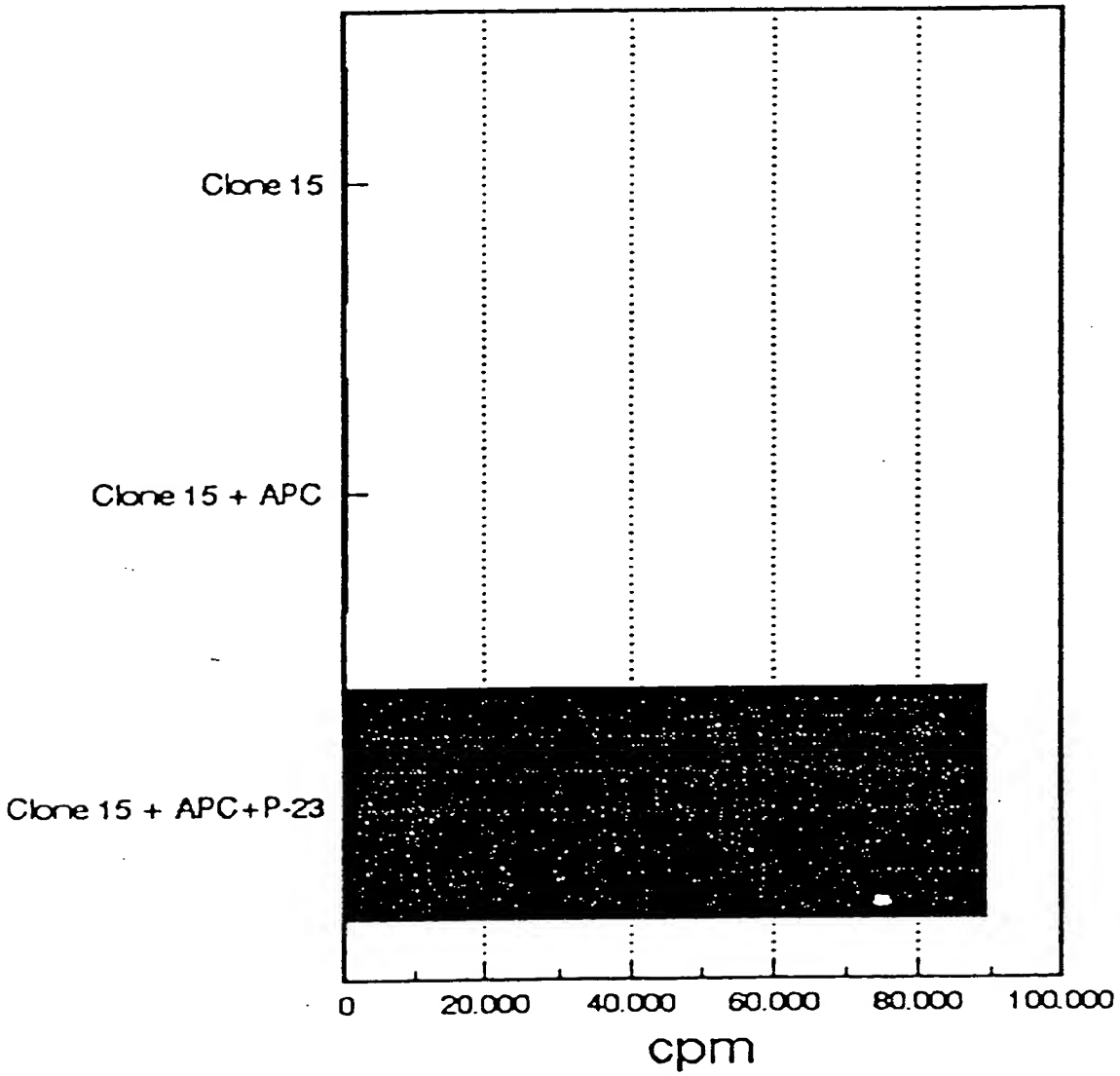


Clone: 50000 cells/well
APC: irradiated allogeneic HLA-DQ identical PBMC 50000 cells/well
Peptide 23: 50 micrograms/ml

Figure 12

Peptide 23 specific clones derived from patient nr.29

(continued...)

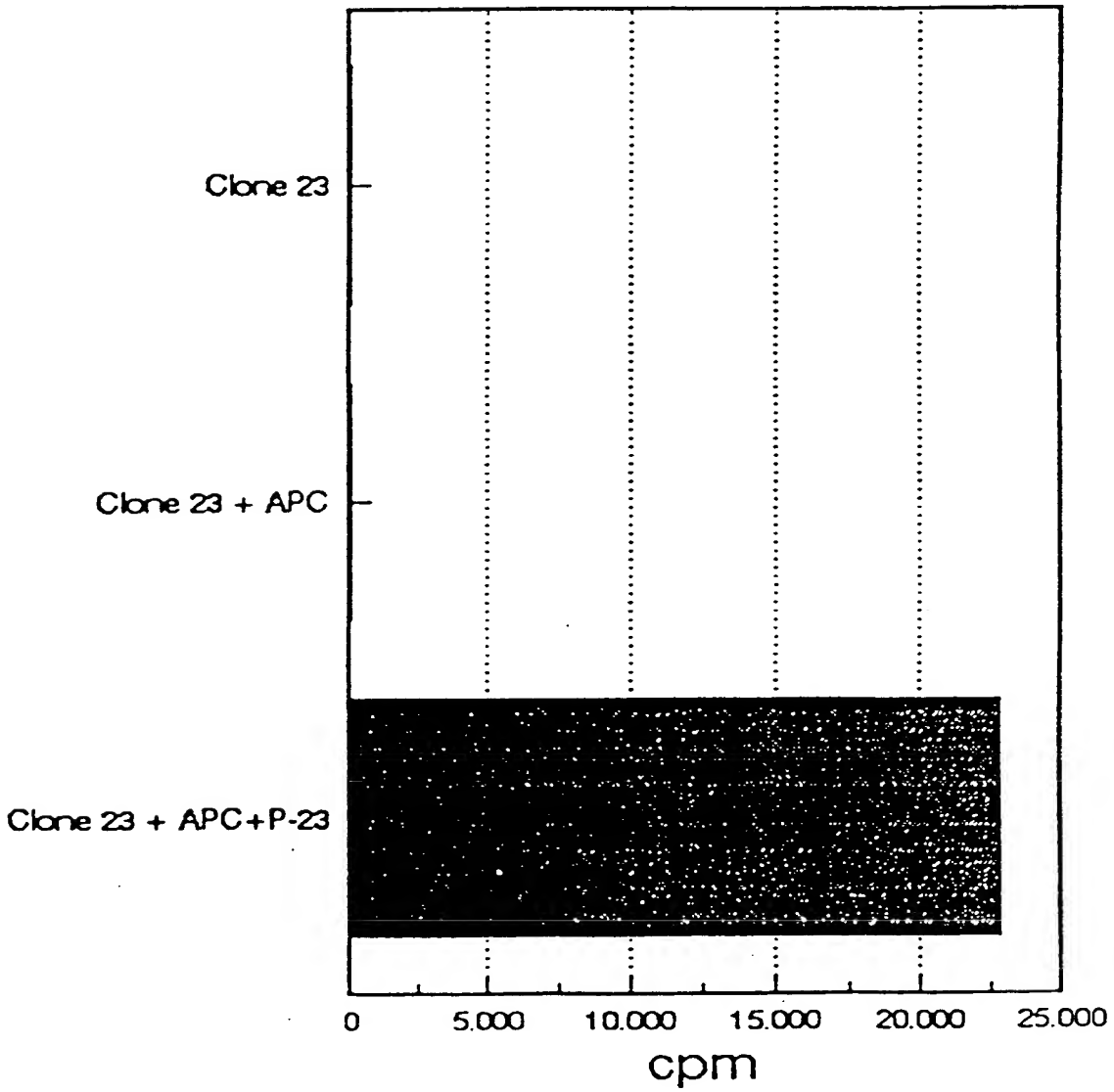


Clone: 50000 cells/well
APC: irradiated allogeneic HLA-DQ identical PBMC 50000 cells/well
Peptide 23: 50 micrograms/ml

Figure 12

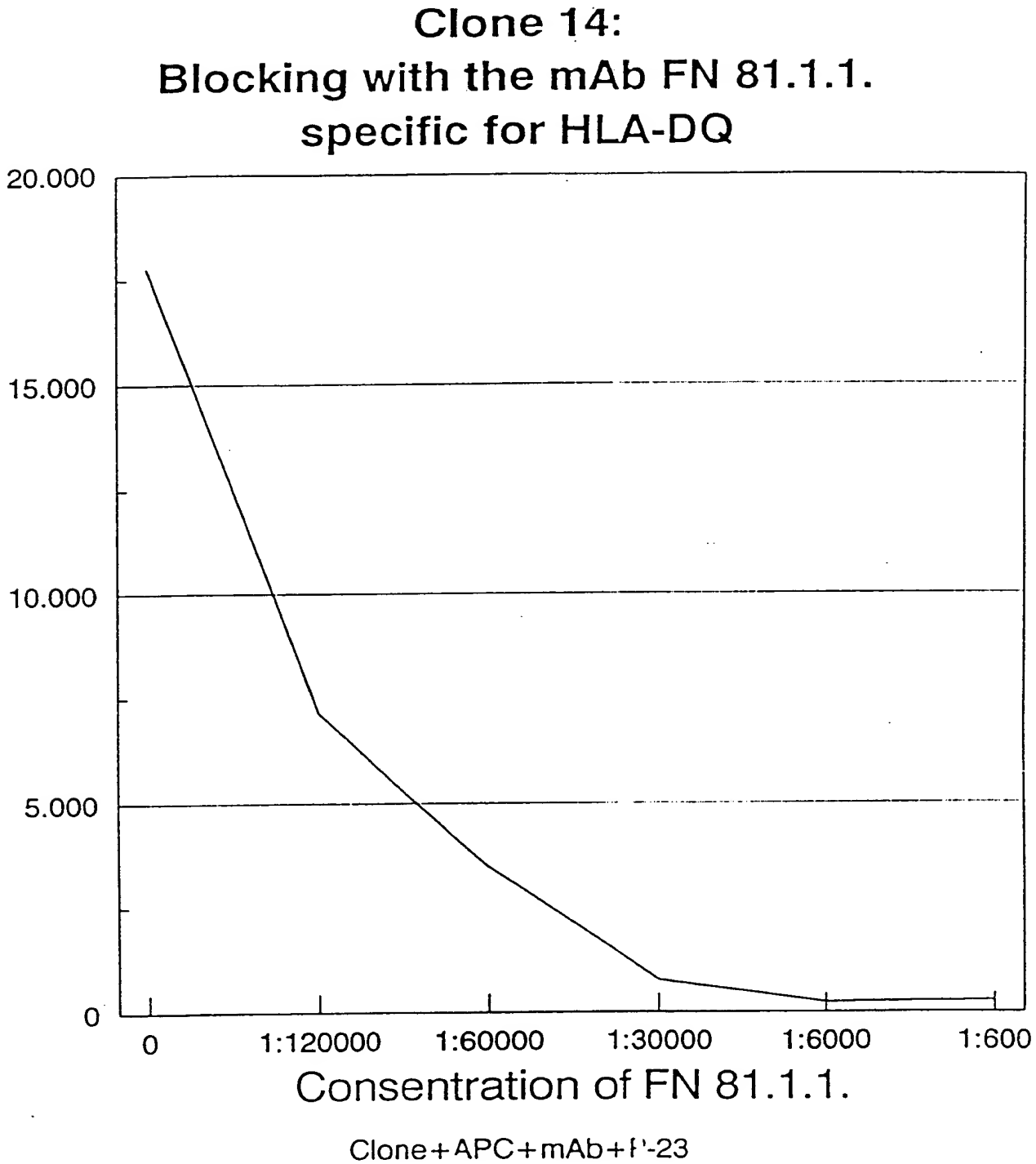
Peptide 23 specific clones derived from patient nr.29

(continued...)



Clone: 50000 cells/well
APC: irradiated allogeneic HLA-DQ identical PBMC 50000 cells/well
Peptide 23: 50 micrograms/ml

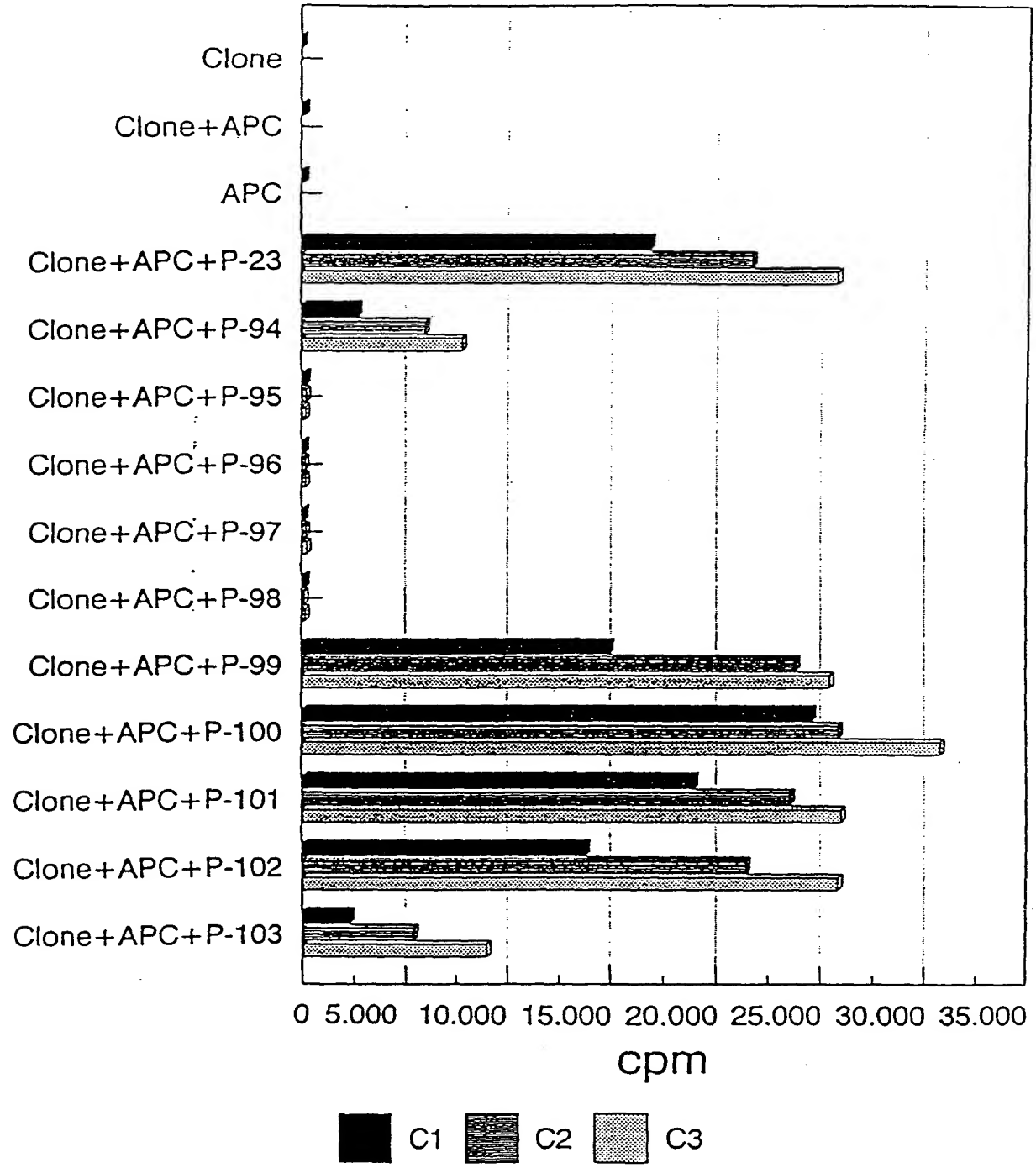
Figure 12



Clone: 50000 cells/well
APC: 50000 cells/well
Consentration of P-23: 50 micrograms/ml

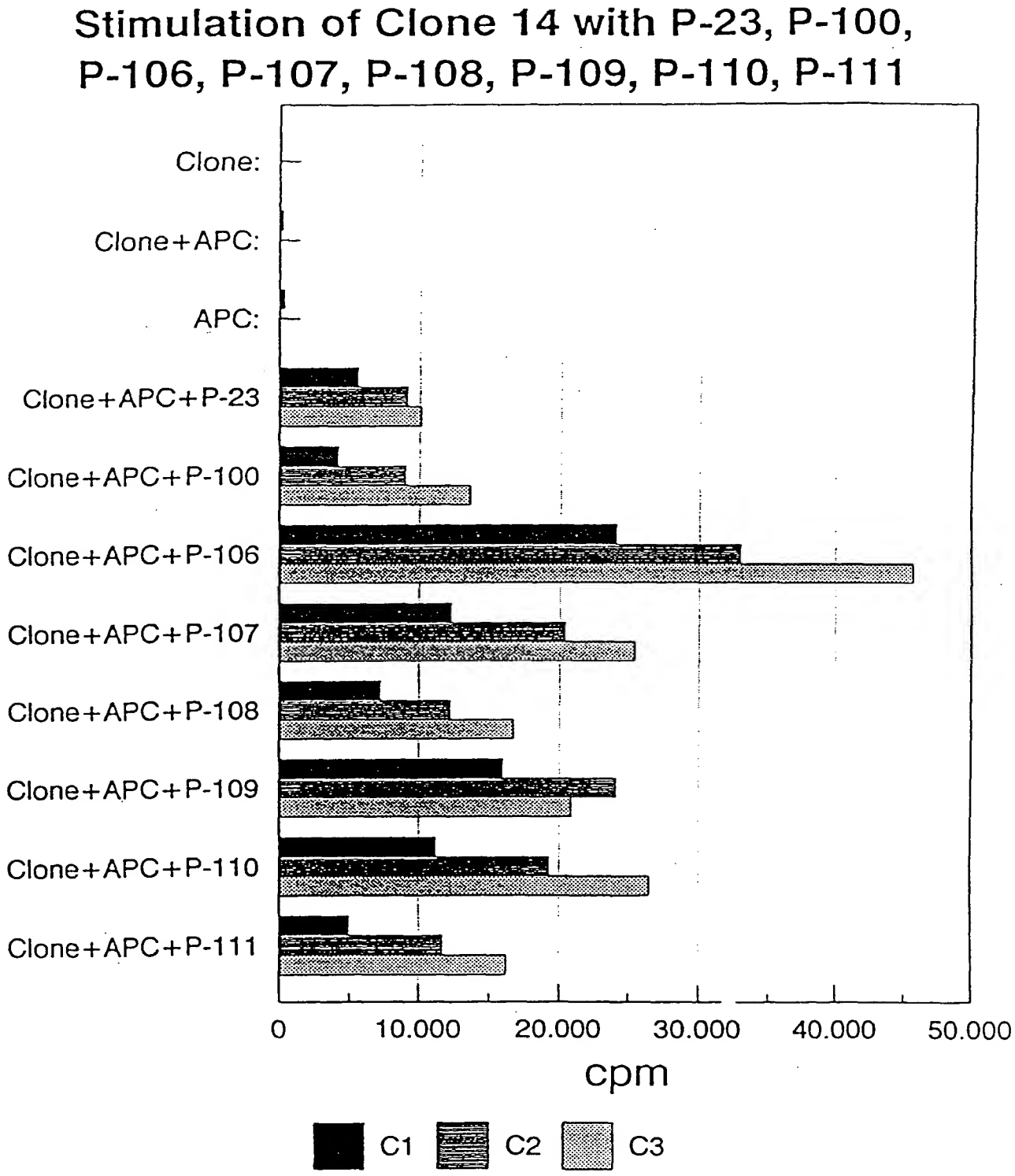
Figure 13

Stimulation of Clone 15 with truncated peptides:



Clone: 5(000 cells/well,APC: 50000 cells/well
C1=20 micro M, C2=50 micro M, C3=100 micro M

Figure 14



Clone: 32000 cells/well, APC: 50000 cells/well
C1= 20 micro M, C2= 50 micro M, C3= 100 micro M

Figure 15

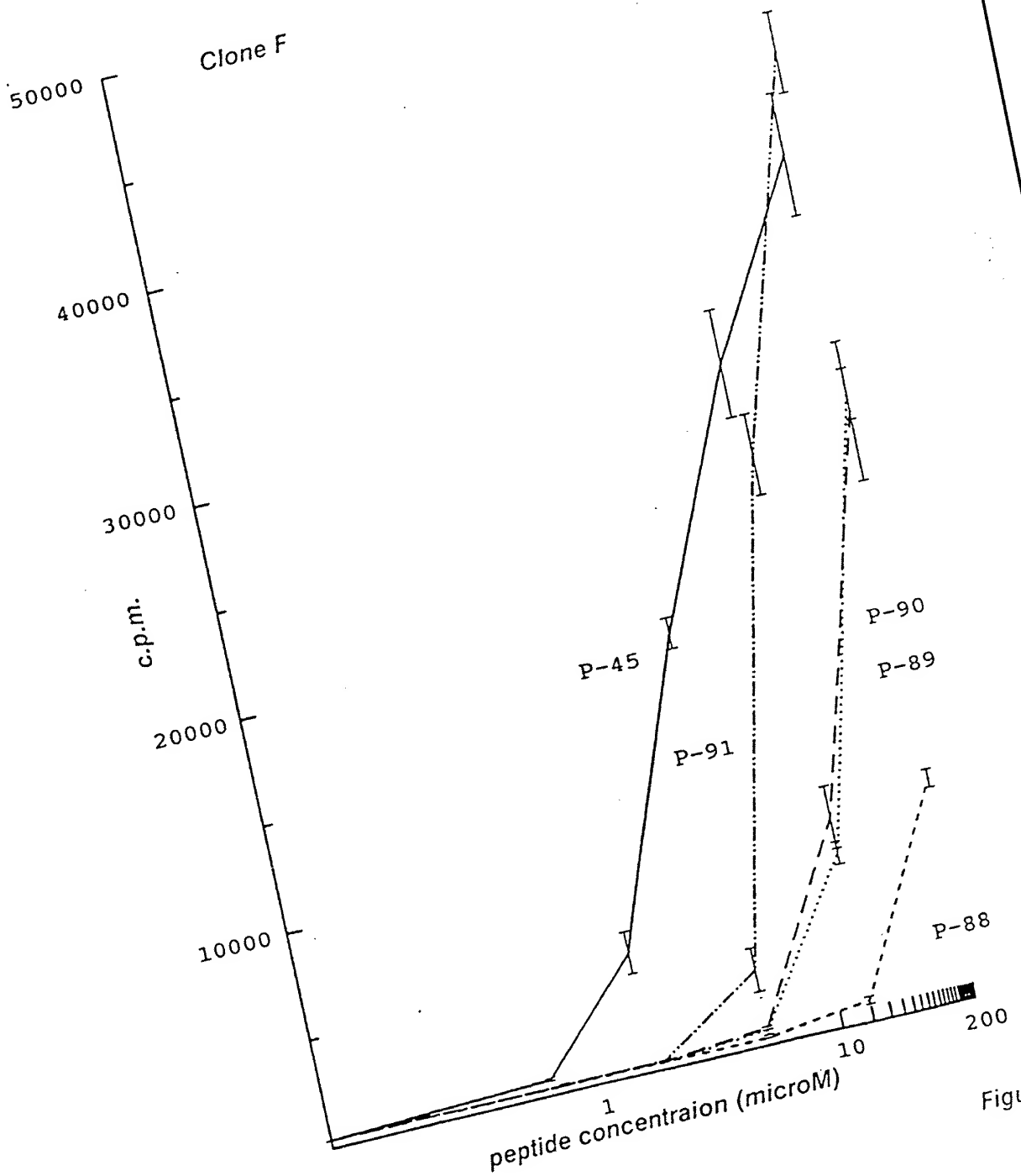

Clone F
50000
40000
30000
20000
10000
c.p.m.
P-45
P-91
P-90
P-89
P-88
1
10
200
peptide concentraion (microM)

Figure 16

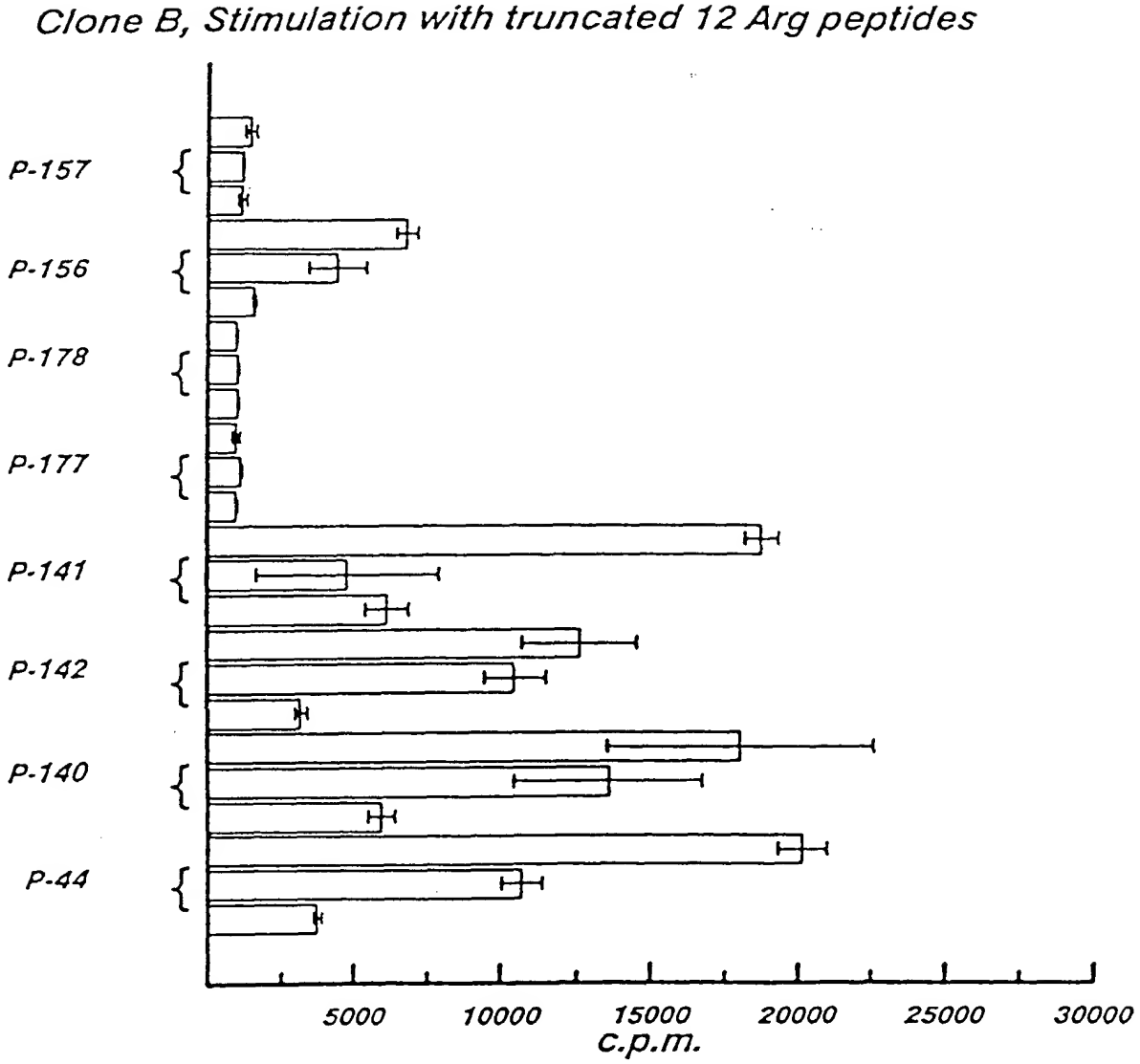

Clone B, Stimulation with truncated 12 Arg peptides
P-157
P-156
P-178
P-177
P-141
P-142
P-140
P-44
5000
10000
15000
20000
25000
30000
c.p.m.

Figure 17

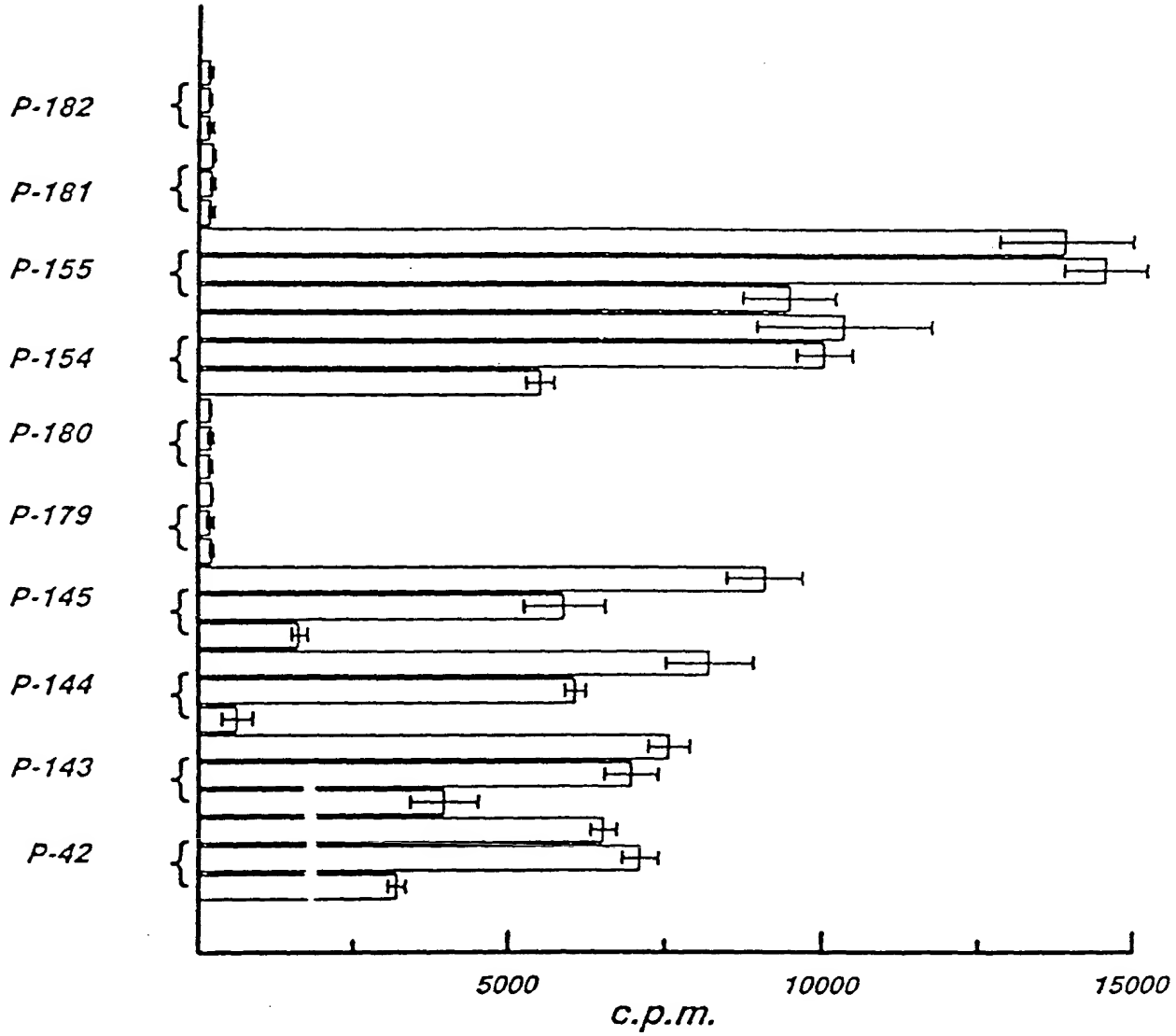


Figure 17

(continued...)

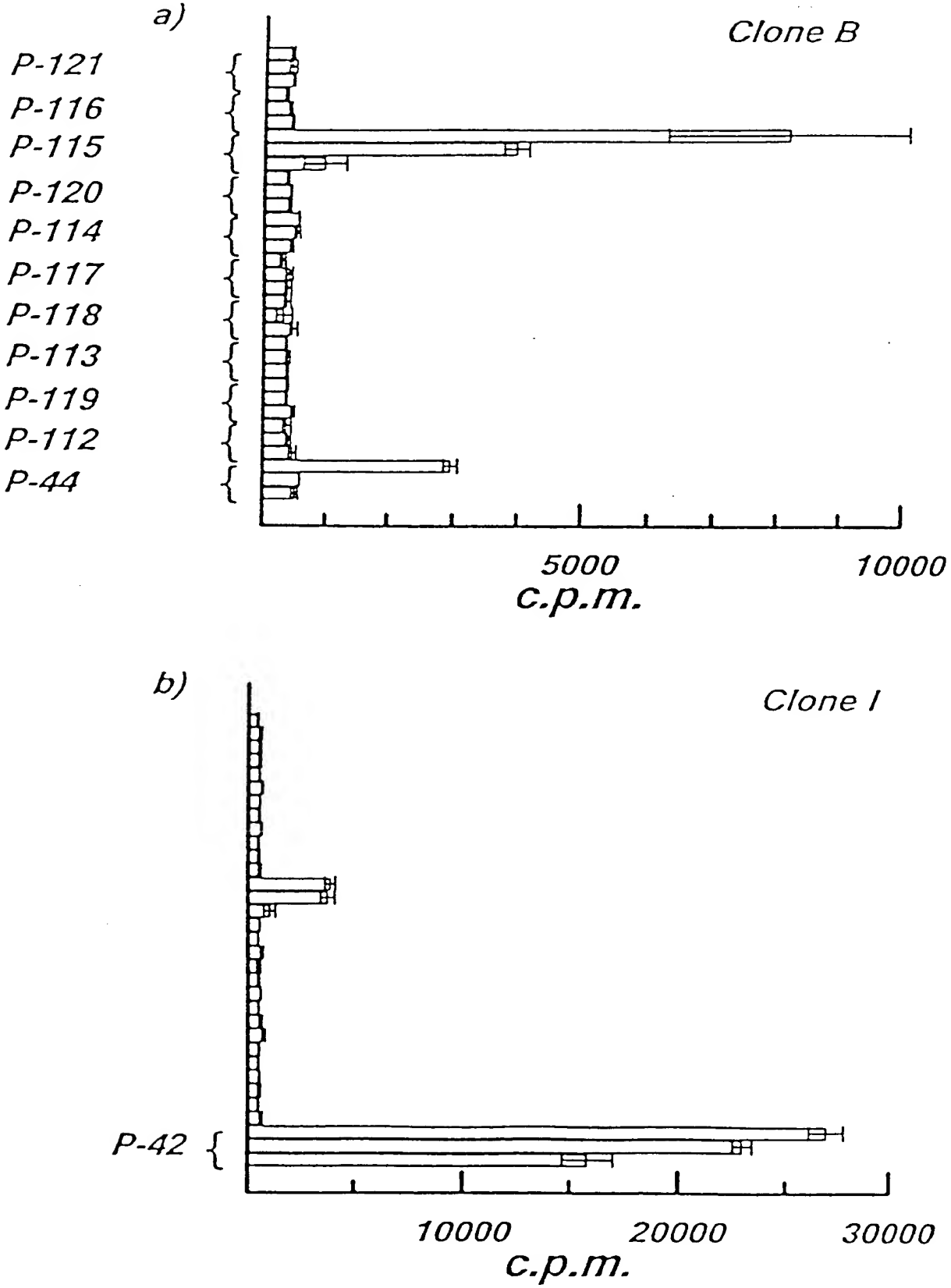
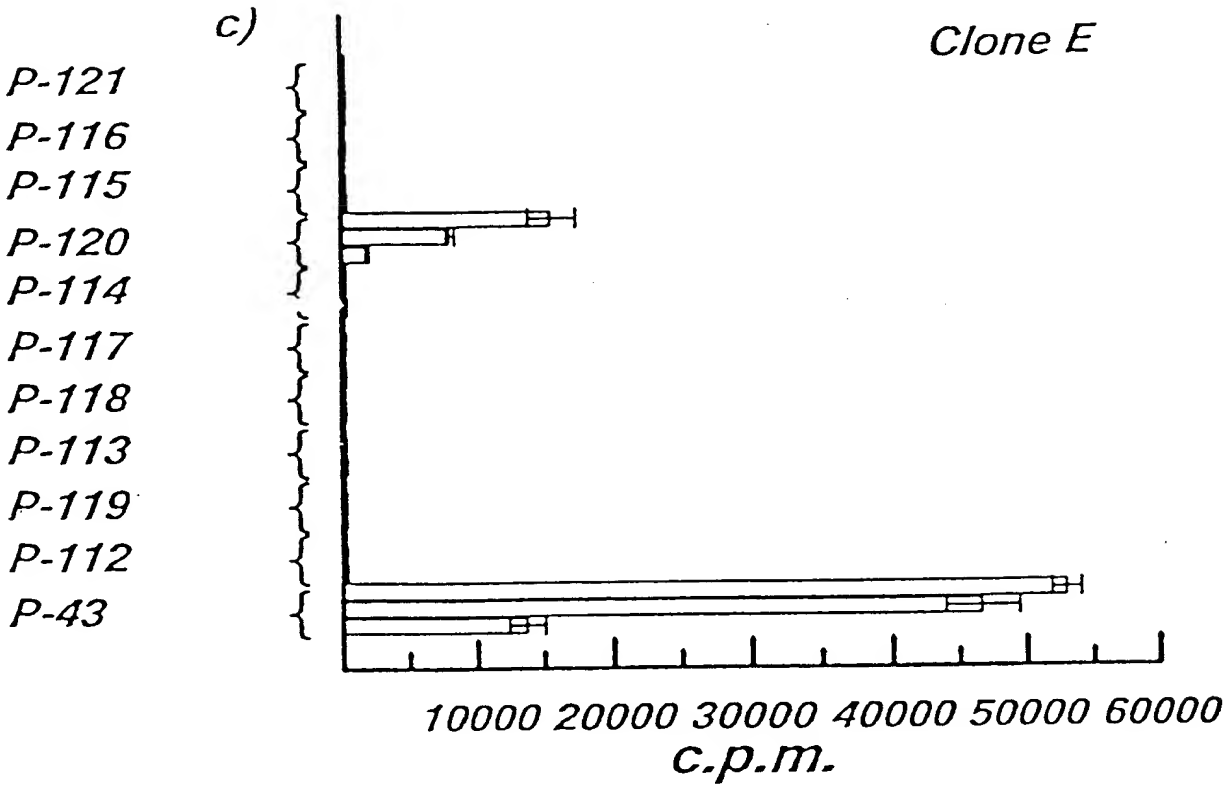

a)
Clone B
P-121
P-116
P-115
P-120
P-114
P-117
P-118
P-113
P-119
P-112
P-44
5000
10000
c.p.m.
b)
Clone I
P-42
10000
20000
30000
c.p.m.

Figure 18



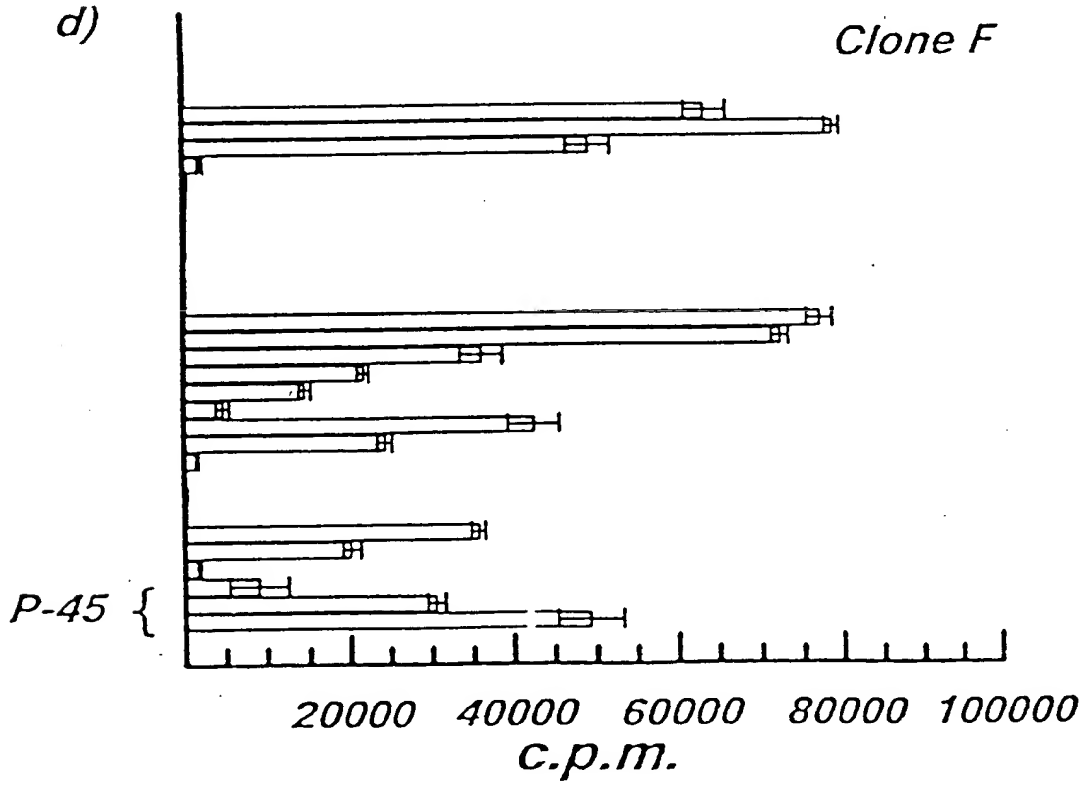


Figure 18

(continued...)

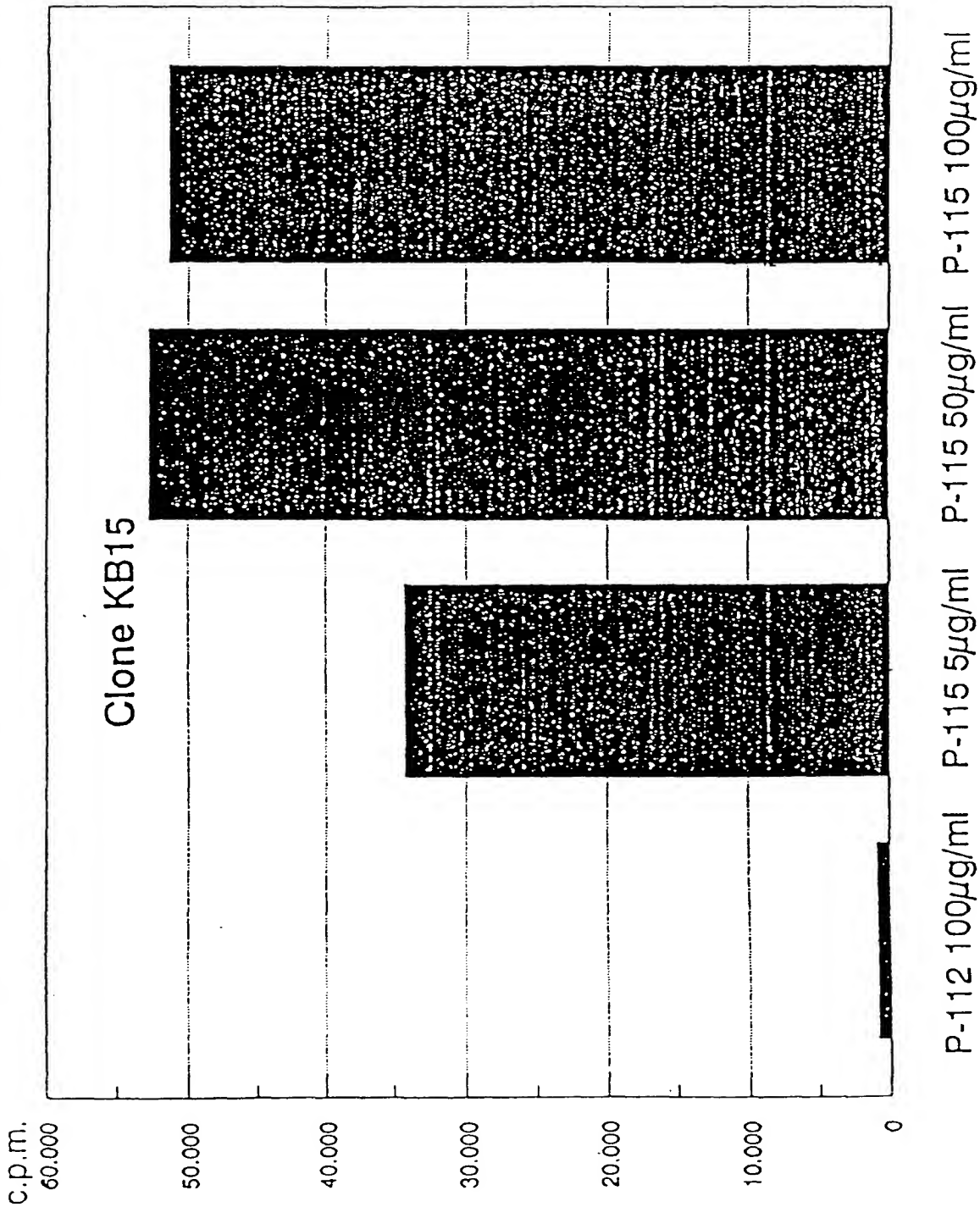

Clone KB15
c.p.m.
60.000
50.000
40.000
30.000
20.000
10.000
0
P-112 100µg/ml
P-115 5µg/ml
P-115 50µg/ml
P-115 100µg/ml

Figure 19

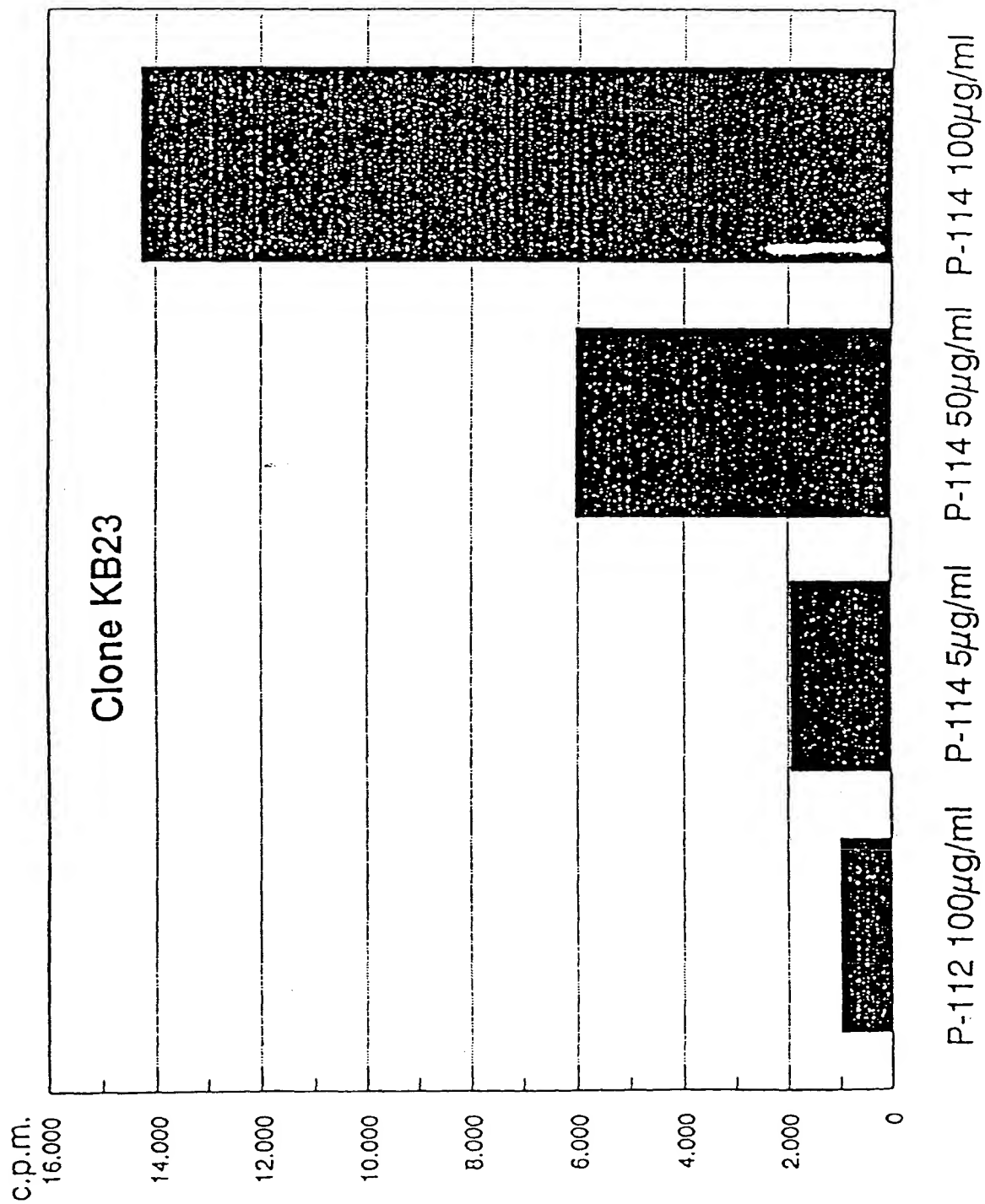
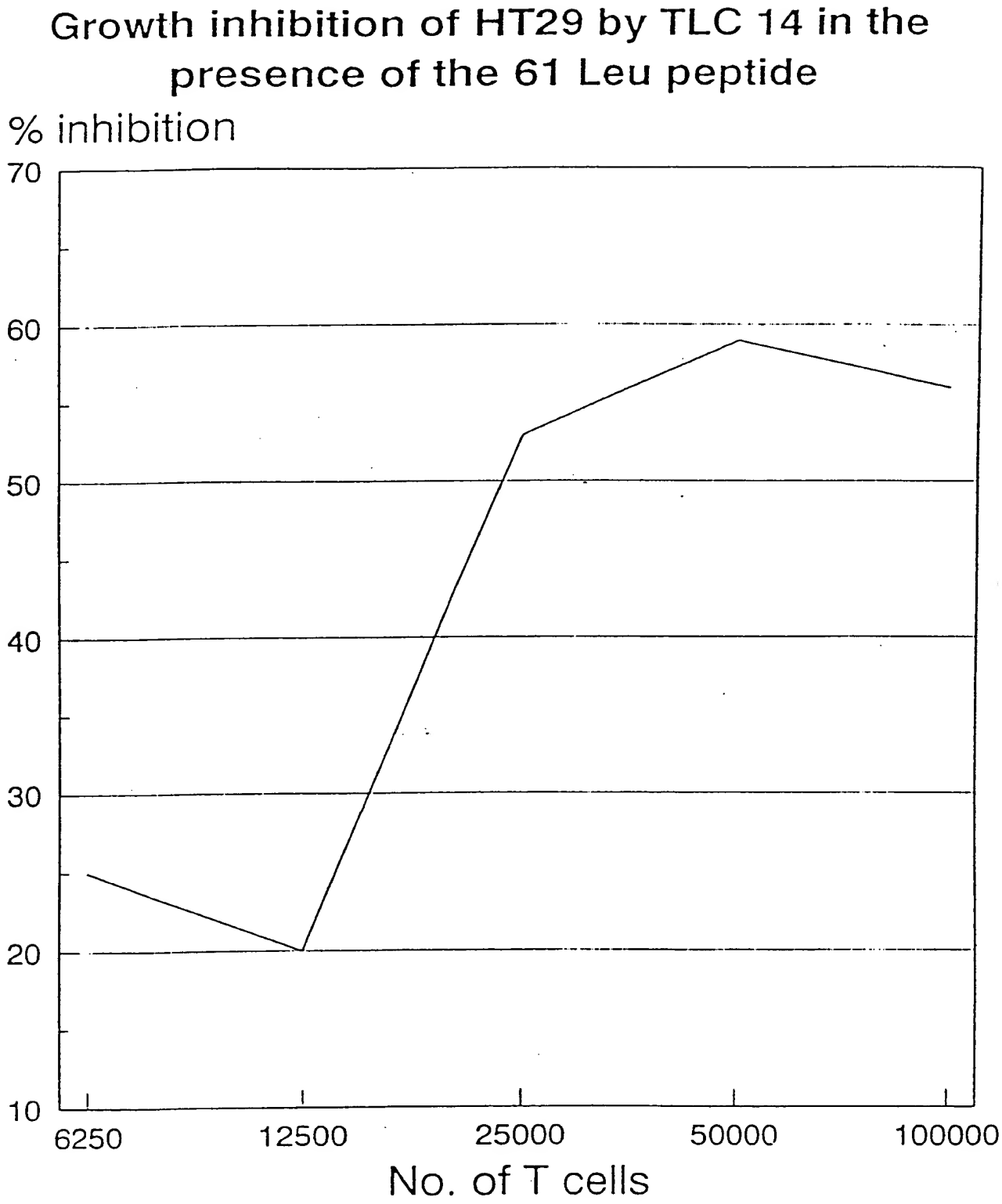


Figure 19

(continued...)



HT29 was preincubated with gamma-IFN for 3 days

Figure 20

INTERNATIONAL SEARCH REPORT

International Application No PCT/NO 92/00032

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC5: C 07 K 13/00, C 07 K 7/10, A 61 K 37/02, A 61 K 39/00

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
IPC5	C 07 K; A 61 K

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in Fields Searched[8]

SE,DK,FI,NO classes as above

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category *	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
X	J.EXP.MED., Vol. 173, 1991 Stefan Jung et al: "Human T Lymphocytes Recognize a Peptide of Single Point-mutated, Oncogenic ras Proteins", see page 273 - page 276 --	1-6
X	WO, A1, 9001701 (PROGENX, INC.) 22 February 1990, see especially table 1 polypeptide no 141, 232, 143, 144, 145, 231 --	1-6
X	US, A, 4898932 (CARNEY) 6 February 1990, see especially the abstract, column 4 lines 20-34 --	1-6
X	WO, A1, 8910565 (E.I. DU PONT DE NEMOURS AND COMPANY) 2 November 1989, see especially page 10 line 14 - page 26 line 10 --	1-6

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance, the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance, the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
5th June 1992	1992 -06- 10
International Searching Authority	**Signature of Authorized Officer**
SWEDISH PATENT OFFICE	Mikael G:son Bergstrand

Form PCT/ISA/210 (second sheet) (January 1985)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
X	WO, A1, 8908710 (E.I. DU PONT DE NEMOURS AND COMPANY) 21 September 1989, see the abstract, claims 1-2 --	1-6
X	WO, A1, 8904489 (PROGENX, INC.) 18 May 1989, see especially table 1 --	1-6
X	EP, A2, 0190033 (E.I. DU PONT DE NEMOURS AND COMPANY) 6 August 1986, see the abstract --	1-6
X	WO, A1, 8500807 (SCRIPPS CLINIC AND RESEARCH FOUNDATION) 28 February 1985, see especially claim 5 --	1-6
X	EP, A1, 0108564 (THE REGENTS OF THE UNIVERSITY OF CALIFORNIA) 16 May 1984, see especially claim 17 --	1-6
X	BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS, Vol. 160, No. 1, 1989 Chien-Hua Niu et al: "Comparison of the conformation and GTP hydrolysing ability of N-terminal ras p 21 protein segments", see page 282 - page 288 see especially page 283 lines 2-7 --	1-6
X	BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS, Vol. 157, No. 2, 1988 P.A. Longo et al: "The structure of the amino terminal transforming segment of the p21 protein, Tyr4-Thr20 (with Asp12), by two-dimensional NMR", see page 776 - page 782 see especially page 777 lines 21-32 --	1-6
X	PROC.NATL.ACAD.SCI., Vol. 82, 1985 Takeo Tanaka et al: "Efficient generation of antibodies to oncoproteins by using synthetic peptide antigens", see page 3400 - page 3404 see especially table 1 --	1-6

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. ☒ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☒ Claim numbers 7-8, because they relate to subject matter not required to be searched by this Authority, namely:

Methods for treatment of the human or animal body c.f. PCT RUle 39.9.

2. ☒ Claim numbers 1, 4-6 because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

These claims relate to peptides derived from unspecific oncogenes having an unspecific point of mutation or translocation. The amount of information in these claims is not sufficient to make a complete search and hence, the claims are considered to be unacceptable according to PCT article 6. The search has been restricted to peptides derived from the p 21 ras protein.

3. ☐ Claim numbers....., because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the the claims. It is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additonal fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional seach fees.

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. PCT/NO 92/00032

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the Swedish Patent Office EDP file on 30/04/92
The Swedish Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

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